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Cadmium modulates expression of vascular alpha 1 adrenoceptors and aortic smooth muscle cells phenotype

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By

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ABSTRACT

Cardiovascular diseases (CVDs) remain the leading cause of death worldwide, including Qatar. Hypertension is one of the most common CVDs that contribute to this mortality. Cadmium (Cd) is a well-known pollutant that has been suggested to be a risk factor for hypertension. However, the underlying mechanisms are still lacking. Very little is known about the effect of Cd on the expression of vascular alpha- 1 adrenoceptors (α1 ARs) in vascular smooth muscle cells (VSMCs). This study was therefore undertaken to determine the effect of Cd on the expression and sensitivity of vascular α1 ARs *in vitro* and *in vivo.* Along with that, there are several phenotypic changes could modulate the VSMCs function and contribute to CVDs including hypertension. These changes include hypertrophy, migration and senescence. The second objective of this study was to determine the effect of Cd on VSMCs phenotype. Human aortic smooth muscle cells (HASMCs) were incubated with different concentrations of cadmium chloride ($CdCl_2$) for varying durations. The results indicated that Cd increases the expression of α1 ARs in HASMCs in a concentration and time dependent manner. To determine if Cd modulates the transcriptional activity of α1 ARs, cells were pre-incubated with actinomycin D, a DNA-dependent RNA synthesis inhibitor. Interestingly, the Cd-induced α1 ARs protein expression was abolished by actinomycin D. Moreover, this expression of α1 ARs was diminished when cells were pre-incubated with H89, a protein kinase A (PKA) inhibitor. This indicates that PKA plays an important role in mediating the Cd-induced

expression of α1 ARs. α1 ARs activity was determined through functional study. Wistar rats were treated with $CdCl_2$ at dose 15mg/kg/day via drinking water for 8 weeks. Blood pressure was measured at the baseline and after 30 days of treatment. Cd didn't affect the vascular reactivity of rat mesenteric arteries in response to α1 ARs agonists, phenylephrine and norepinephrine (NE) as well as blood pressure. Antagonizing α1 ARs with prazosin didn't show any response of other alpha's AR. To determine the effect of Cd on VSMCs phenotype, HASMCs were incubated with $CdCl_2$. Our results showed that cadmium induces hypertrophy, migration and senescence. Taken together, our results dissect a novel pathway employed by Cd to increase expression (not the activity) of vascular α1 ARs, a major player in hypertension as well as VSMCs phenotypic modulation. This new paradigm offers a better understanding and thus potential amelioration of pollution-related CVDs.

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CHAPTER 1: INTRODUCTION

Environmental pollution

Environmental pollution is considered to be one of the critical concerns that has a significant impact on human health. Rapid growing, urbanization, industrial activities and abuse of available resources play a role in destroying and polluting the environment. These anthropogenic processes spread environmental pollutants everywhere: in air, soil and water. As such humans are becoming highly exposed to these pollutants, which may lead to the development of serious health problems. Recently, the World Health Organization (WHO) reported that 23% of the global disease burden is attributable to the environment (1). In fact around two million people died of complications that resulted from exposure to indoor air pollution, and the number of deaths resulting from diarrhea caused by the consumption of unsafe contaminated water reached 88% (1). In order to prevent or minimize the increment in these statistical data, a well-planed environmental management program is key to avoid illnesses caused by the abovementioned introduced environmental factors. It is estimated that implementing such a program and preventing or minimizing environmental pollution risk can actually save more than four million lives per year, the majority of which are in developing countries (1).

Among the many factors that contribute to environmental pollution are heavy metals, which are characterized as having a density higher than 4.5 g/cm3 (2). Heavy metals can naturally be found within a range of background concentration in the environment; some examples of heavy metals include: lead, mercury, cadmium, arsenic,

chromium, zinc, nickel and copper. Anthropogenic activities can produce a higher concentration of these metals than the normal existing level. Such anthropogenic activities include plastic and rubber industries, metal smelting and refining industries, scrap metal, burning of elements-containing wastes and various consumer products (3). Therefore, changes of the concentration levels of these metals beyond the range of the background, increase the risk of environmental pollution, and unlike other types of pollutants, heavy metals are not easily removed from the environment and can persist in the environment for a very long time. Once released, heavy metals can travel to different environmental compartments and find their way to the food chain. Accordingly, the Protocol on Heavy Metals to the Convention on Long-Range Transboundary Air Pollution (CLRTAP) was adopted on 24 June 1998 in Aarhus (Denmark) to target three harmful metals, which are cadmium, lead and mercury. This protocol was amended in 2012 to implement more strict regulations on heavy metals emissions (2).

Cadmium

Cadmium (Cd) is a naturally occurring heavy metal, which exist in the environment with a high toxicity. Cadmium has been listed as No 7 out of 275 most hazardous materials (ATSDR 2011) according to the Comprehensive Environmental Response, Compensation and liability (CERCLA) (4). Pure Cd is a soft, silver-white metal with a melting point of 320.9°C. Chemically it has the symbol Cd and atomic number 48. However, it is not easy to be found in nature as pure form, it is usually occurred in combination with other elements such as sulphur, which form cadmium sulphate, oxygen which form cadmium oxide and chlorine which form cadmium chloride

(5).

Cd can be released to the environment as a result of volcanic activities and industrial activities. Cd is widely used in industrial processes, e.g.: as an anticorrosive agent, as a stabilizer in polyvinyl chloride (PVC) products, as a colour pigment, a neutron-absorber in nuclear power plants, and in the fabrication of nickel-cadmium batteries as well as phosphate fertilizers which showed a big cadmium load (6). Cd can also be found in tobacco smoke (7). Cd level increased during the 20th century, one of the reasons is miscarry to get rid of Cd-containing products, this level started to decrease by the end of the 20th century due to stringent environmental legislation (8). In developing countries environmental Cd availability increased due to industrial processes and solid waste incineration (9). As a result, Cd particles reach soil and water through dry and wet decomposition. Once these particles settled down, they are taken up by plants and other organisms thus entering the food chain. In 1963 WHO international standard for drinking water recommended the allowable concentration of Cd intake to be 0.01 mg/litre (10). In 1984 a new value was published in the first edition of the Guidelines for Drinking-water Quality, which was 0.005 mg/litre (10). This value was then decreased to reach 0.003 mg/litre of water in the 1993 Guidelines based on the PTWI set by JECFA (10). All of these guidelines and regulations were established in order to reduce the availability of this toxic metal in water. The implementation of such straight regulations help to reduce Cd emissions in some developed countries (fig.1) (11). Cd concentration has been declined as shown in some developed countries, but the problem is that Cd exists in the environment with a high toxicity. It is toxic at very low exposure levels and has acute and

chronic effects on health and environment (12). The first documented incidence related to Cd toxicity was *itai itai* disease reported in Japan after the Second World War (13). This disease appeared to result from consumption of rice irrigated with Cd-contaminated water. Since then, increasing attention has been given to cadmium and its effects on human health.

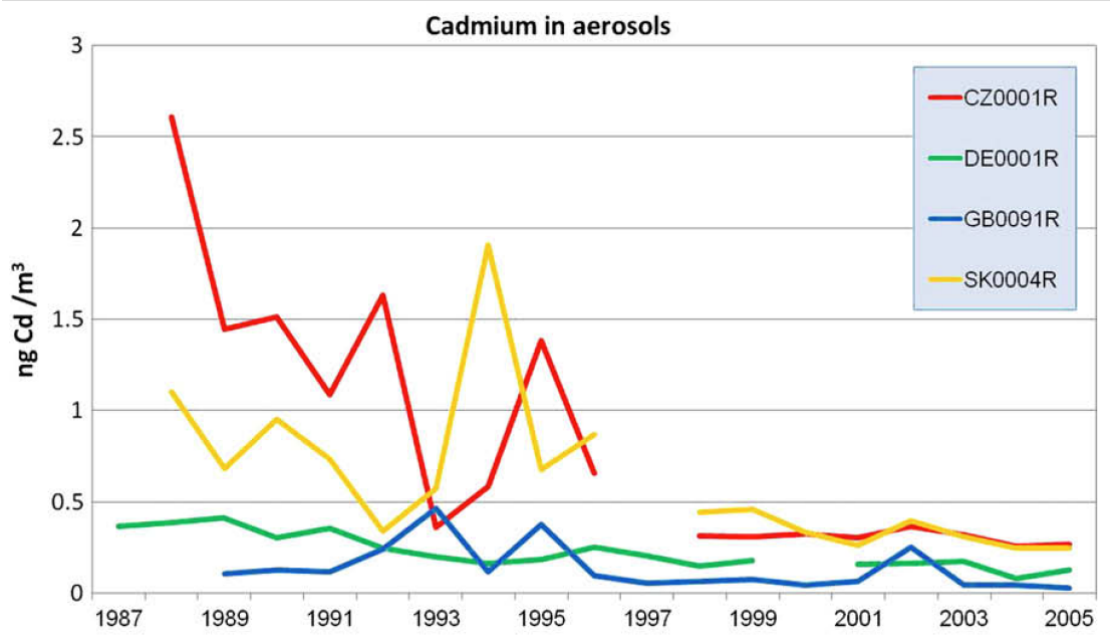


Figure 18: Change of air concentrations of Cd at selected stations in Europe (CZ:Czech Republic, DE: Germany, GB: Great Britain, SK: Slovakia) from 1987 through 2005 (in ng m^{-3}) (11).

Human Exposure to Cadmium

Since Cd is spread in the environment, humans are highly exposed to this pollutant. It can enter the human body through different routes such as: gastrointestinal, pulmonary and dermal contact. The main source for the gastrointestinal exposure is Cd-contaminated food. Once Cd reaches soil it persists for along time in which it is taken up

by the plants and enter the food chain to target the human body. Cd-contaminated food can include animals such as goats, sheep, cows and chickens, which feed on the naturally contaminated sources. Also, seafood can be contaminated with Cd if they come from contaminated seas and oceans. The amounts of Cd ingested daily with food in most countries are in the range of 10 to 20 μg per day (7).

Inhalation of Cd mostly affects workers in the industrial areas and people who are living near to those areas. Tobacco smoke contributes to inhalation exposure for smokers. Since one cigarette contains approximately 1 to 2 μg Cd, smoking one pack per day results in a daily uptake of Cd that approximates that derived from food (7). Workers who are working in the industrial areas can ingest Cd through contaminated hands.

Dermal absorption occurs when the human skin get in contact directly with contaminated soil or water. Cd by this way penetrates the dermal cells to reach the blood. Two mechanisms facilitate cadmium absorption by the skin: binding of a free cadmium ion to sulfhydryl radicals of cysteine in epidermal keratins, or an induction and complexing with metallothionein (MT) (6).

Cadmium metabolism

Upon absorption, Cd ions cross various barriers and reach the bloodstream. There it binds to red blood cells or high molecular weight proteins such as albumin and metallothionein (MT) (14). Cd is transported through the blood stream and accumulated in body organs especially kidney and liver, with 50-80% of body burden being stored in kidney and liver (15). When cadmium reaches the liver it stimulates the production of MT, a protein that has a major role in Cd detoxification. MT then starts to bind to free Cd

to reduce toxicity. Cd complexes with MT then leaves the liver and arrives at the kidney to be cleared through glomerular filtration. After filtration Cd is taken up by the proximal tubule where the bond between Cd and MT is cleaved and free Cd released. These free Cd start to induce the production of MT again in the kidney. MT production in the kidney is lower and inadequate to bind to free Cd. Free Cd then interacts with the kidney cells leading to kidney damage (15). Importantly, Cd has a very long biological half-life 15-30 years (16).

Cardiovascular Diseases

Cardiovascular diseases (CVDs) remain the leading cause of morbidity and mortality in developing and developed countries (17) .In 2004 alone, around 17.1 million people died because of CVDs. This number is estimated to increase and reach around 23.6 million by 2030 (18).

Hypertension is one of the CVDs that is considered an endemic problem (19). In fact, hypertension remains the most powerful risk factor for CVDs (20). It is the most chronic disease that affects one billion of human population worldwide (21). Hypertension and hypercholesterolemia are responsible for around 50-75% of all CVDs (18). The American Heart Association redefined hypertensive to be pre-hypertension when the blood pressure reaches 120 to 139 mmHg over 80 to 89 mmHg, and hypertension when blood pressure is 140 mmHg over 90 mmHg or above (22). Several risk factors are associated with hypertension including smoking, diet, lifestyle, genetic factors and physical inactivity. In addition to these factors, environmental pollution contributes in inducing hypertension. Recently, increasing evidence-highlighted cadmium

to be one of these contributors. Epidemiological studies found that there is a correlation between blood level of cadmium and blood pressure elevation (16). More supportive evidence showed that animal treated with $CdCl_2$ also modulate blood pressure and induce hypertension (23) (24) (25).

The regulation of blood pressure is subject to various regulatory influences. Indeed, many regulators are integrated, so that a blood pressure homeostasis is maintained. These includes: the vasculature, kidneys, sympathetic and central nervous system as well as their hormonal regulators (26). Hormonal regulation requires interaction between the hormones and specific receptor that are mostly distributed on the cell membranes. These hormones are the active substance that promote cell-signaling cascade when they bind to its receptor. In pharmacology, these receptors are the target for therapeutic agents. One of the most important receptors that contribute to the generation of vascular tone is adrenergic receptor

Adrenergic receptors are a G-protein coupled transmembrane receptors (GPCRs) that are divided into three families: α1 (α1A, α1B, α1D), α2 (α2A, α2B, α2C) and β (β1, β2, β3) (27). These receptors are sensitive to catecholamines: epinephrine (EP) and norepinephrine (NE). Each type and class of these receptors is responsible for specific function based on its location and distribution among body tissues (Table.1) (28).

Table 1: Distribution and Response of Adrenergic Receptor Subtypes (28).

Receptor	Physiology	
	Tissue	**Response**
$\alpha_{1A,B,D}$	Smooth muscle: vascular, iris, radial ureter, pilomotor, uterus, sphincters	Contraction

	(gut, bladder)	
	Smooth muscle (gut)	Relaxation
	Heart	Positive inotropic ($\beta_1 >> \alpha_1$), cell growth, hypertrophy
	Salivary gland	Secretion
	Adipose tissue	Glycogenolysis
	Sweat glands	Secretion
	Kidney (proximal tubule)	Gluconeogenesis, Na^+ reabsorption
$\alpha_{2A,B,C}$	Presynaptic autoreceptor on sympathetic nerve endings	Inhibition of norepinephrine release
	Platelets	Aggregation, granule release
	Endocrine pancreas	Inhibition of insulin release
	Adipose tissue	Inhibition of lipolysis
	Vascular smooth tissue	Contraction
	Kidney	Inhibition of renin release
β_1	Heart	Positive inotropic effect, positive chronotropic effect, cell growth, hypertrophy
	Adipose tissue	Lipolysis
	Kidney	Renin release
β_2	Liver	Glycogenolysis, gluconeogenesis
	Skeletal muscle	Glycogenolysis, lactate release
	Smooth muscle: bronchi, uterus, gut, vascular (skeletal muscle), detrusor	Relaxation
β_3	Endocrine pancreas	Insulin secretion
	Salivary gland	Amylase secretion

	Adipose tissue	Lipolysis
	Striated muscle	Thermogenesis

Alpha 1 Adrenergic Receptors (α1 ARs)

α1 ARs are the most involved type in smooth muscle cells contraction. These receptors are highly expressed in the vascular smooth muscle cells (VSMCs) and are responsible of VSMCs contraction. Due to this, α1 ARs have an important role in the vasculature. Adrenergic receptor-induced contraction requires an interaction between the receptor and EP or NE. This interaction activates the receptor to undergo signaling transduction. Upon activation, $G_{q/11}$ protein dissociates to stimulate phospholipase C causing increase in Inositol triphosphate (IP3) and release calcium ions (Ca^{2+}). This leads to activation of protein kinase C and end with VSMC contraction (Fig.2).

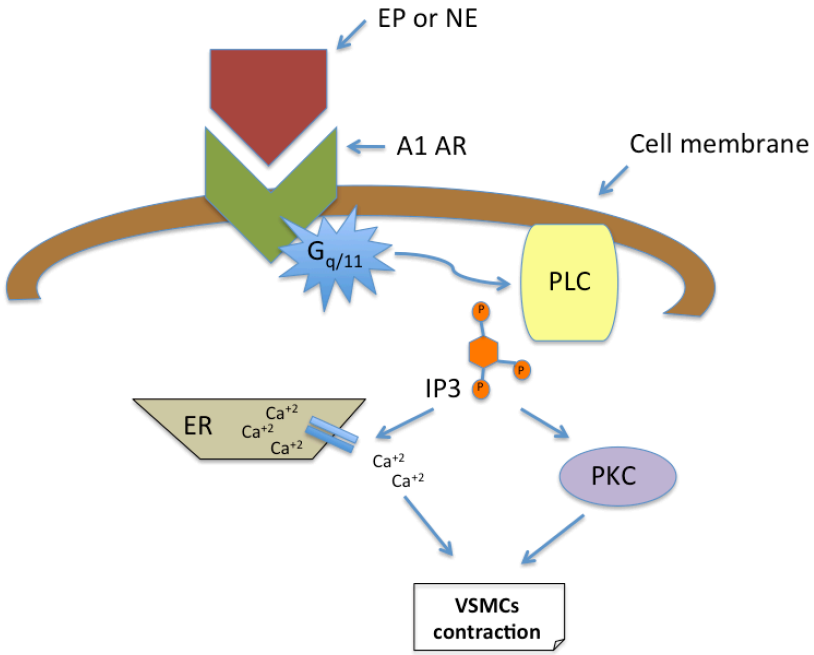


Figure 2: Activation of α1 AR and intracellular signaling cascade.

The physiological function of α1 AR varies according to its expression in various tissues and organs. It has a different role in several locations among body tissues and organs as shown in table.2:

Table 2: Physiologic Function of α1 AR (27).

Organ	Physiologic response	Subtype
Heart: Myocardium	Inotrophy	α_{1a}, α_{1b}
	Hypertrophy	α_{1a}
	Pre-conditioning	α_{1a}
Conduction system	Arrhythmias, bradycardia	?
Brain	Alertness	?
Vessels	Contraction (artery and vein)	$\alpha_{1a}>\alpha_{1b}>\alpha_{1d}$

Prostate	Prostate smooth muscle tone	α_{1a}
Bladder (detrusor)	Irritability	α_{1d}
Gut, gall bladder	Contraction	?

Regulators of α_1 AR activity

In the normal condition, the concentration level of circulating EP and NE is highly controlled in order to maintain homeostasis. Two important mechanisms are involved; one of them is enzymatic degradation by Catechol-*O*-methyltransferase enzyme (COMT). This enzyme acts to inactivate the excess of catecholamines such as Dopamine, EP and NE. The second pathway is the presence of presynaptic α2 AR at the nerve ends. When there is overload of NE secretion, NE binds to the receptor to inhibit the release of this hormone. Therefore, any abnormality in these two mechanisms will cause a disruption in the vascular tone, where high or low blood pressure is expected.

Characteristics of VSMCs Phenotypic Modulation

VSMCs form the medial layer of the blood vessels that support the vasculature. Interestingly, these cells are contract in response to stimulator to control the blood flow and pressure. Indeed, in normal physiological condition most of VSMCs in the vascular wall characterized by spindle-shape, high contractility and low proliferation rate (contractile phenotype) (29). However, in response to injury the contractile phenotype of VSMCs is switched to a synthetic phenotype via a process called phenotypic modulation. Phenotypic modulation is a process in which VSMCs loss their contraction property and characterized by a decrease in SMC differentiation marker gene expression and elevation

in proliferation, migration and matrix synthesis (30). VSMCs phenotypic modulation may be induced by numerous environmental stimuli such as growth factors, inflammatory mediators, mitogens and mechanical influences (31). Under these circumstances, VSMCs remodeling contribute to the development of CVDs such as atherosclerosis, restenosis, aneurysm as well as hypertension (32).

Beside AR-induced hypertension, phenotypic modulation has a role in disrupting the vascular tone and progression of blood pressure. For example, changes in VSMCs phenotype could alter the wall to lumen ratio, decreasing inner diameter and causing blood pressure resistant (Fig.3). Our study highlighted hypertrophy, migration, senescence and viability as examples of VSMCs phenotypic modulations that could affect the blood pressure.

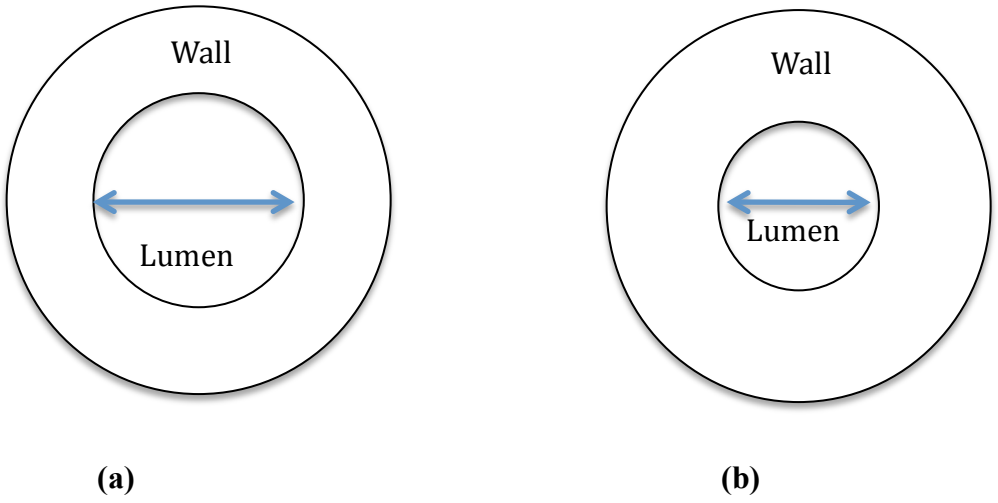


Figure 3: Representative comparison of lumen diameter between normal vessel (a) and vessel after phenotypic modulation (b).

VSMCs hypertrophy is a process results in increase in VSMCs mass within the blood vessels. Cell enlargement occurs due to a consequence of events and mechanisms. These events include increase in intracellular protein synthesis and decrease in protein degradation. Increase water content may also cause an increase in cell volume due to disruption in water and ion exchange. In addition to these two mechanisms, DNA synthesis also could lead to cell hypertrophy. The increase in the cell volume leads to increase in the wall/lumen ration (decrease lumen diameter). This phenomenon could be initiated due to several factors such as growth factors, reactive oxygen species and G-coupled protein receptors (GPCRs) agonist.

During the phenotypic modulation, migration and proliferation are common. Migration is a normal physiologic process occurs during the vascular development and vascular repair in injured tissues. It is the movement of the cells from adventitia to the intimal layer and the formation of neointima. Once migrated cells reach that site they start to proliferate as a sequence of repair process. Proliferation of VSMCs is considered a central event of the repair process (33). The movement and proliferation of VSMCs are initiated primarily via growth factors that activate cell surface receptors (34). Upon activation, VSMCs undergo a sequence of signaling transduction to promote cell migration and proliferation. At this stage cells reduced the expression of proteins that are responsible for contractility (35). Hence, VSMCs works to control vascular repair instead of vascular contraction. Pathologically, these processes are well-known in atherosclerosis that contribute in the formation of atherosclerotic plaque (36). Atherosclerotic plaque and

the formation of the neointima modulate the vascular wall causing lumen narrowing (fig.4).

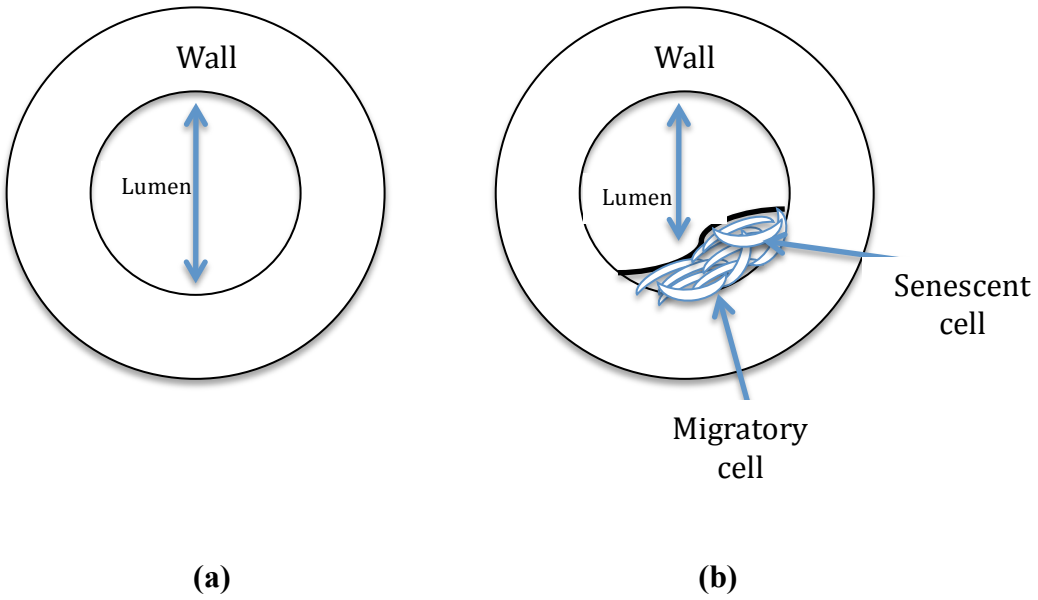


Figure 4: Illustration of lumen diameter in normal condition (a) and after the formation of neointima characterized by migratory and senescent cell (b).

Vascular senescence is another type of phenotypic modulation that is commonly found in the neointima (Fig.4). Vascular senescence is characterized by a permanent cell cycle arrest involving cellular and gene expression changes (37). This phenotype of VSMCs is normally found in aged arteries; hence it is a mark for mammalian aging (37). Moreover, senescent cells are one of the key players in the pathogenesis of atherosclerosis (38). It can be characterized by increased NADPH oxidase activity, high production of reactive oxygen species, low catalase, high expression of cell cycle

progression regulator proteins at stage G1 (p21 and p53) and increase in β-galactosidase activity (39).

LITERATURE REVIEW

Cadmium (Cd) is a natural element of the earth crust that attributes to environmental pollution. As mentioned earlier, several guidelines were established in order to control the spread of this toxic contaminant. The latest update on these guidelines was established by WHO to be 0.003 mg/liter as a safe water daily intake level for human. The toxic effect of Cd is varied among species. In rats and mice for example, the lethal acute oral dose (LD_{50}) is between 60 to 5000 mg/kg body weight (15). While in humans it ranges between 350-3500 mg of Cd (40). Route of exposure are varied, but the most serious significant one is through dietary intake (41) as well as smoking (42). Smokers receive the double daily dose of Cd compared to non-smokers due to smoking behavior (41).

In human, food is the main source of Cd exposure through consumption of food, such as vegetables and grains that are grown in contaminated areas, or seafood originated from contaminated seas and oceans. Table. 3 summaries Cd level in a list of food and estimated exposure rate (43).

Table 3: Cd levels in food and estimates of dietary Cd exposure (adapted from Galal-Gorchev, 1993; Rayment, 1995; Food and Agriculture Organization/World Health Organization, 1998; Resources Sciences, 1997; Australia New Zealand Food Authority, 1998) (43).

	Cd level (mg/kg)				
Food item	Maximum	Typical	Intake level (g/d)	Extreme exposure (μg/d)	Typical exposure (μg/d)
Vegetables, including	0.1	0.05	250	25	12.5

potatoes					
Cereals, pulses and legume, including rice and wheat grain	0.2	0.05	200	40	10
Fruit	0.05	0.01	150	7.5	1.5
Oilseeds and cocoa beans	1	0.5	1	1	0.5
Meat of cattle, poultry, pigs and sheep	0.1	0.02	150	15	3
Liver of cattle, poultry, pigs and sheep	0.5	0.1	5	2.5	0.5
Kidney of cattle, poultry, pigs and sheep	2	0.5	1	2	0.5
Fish	0.05	0.02	30	1.5	0.6
Crustaceans, molluscs	2	0.25	3	6	0.75
Total				93.5	30

Qatar is one of the countries that depends on marine life for food consumption due to its location, where the sea is surround it from 3 sides. As Qatar encounters huge industrial activities during the last five years, it is expected that Cd deposition contaminate marine life. Because the two main industrial areas in Qatar are located in the coast, this may lead to the contamination of seawater. Consequently local fish and shrimp may accumulate the Cd in their body and reached the human body through the food chain and consumption of contaminated seafood. Few studies were carried out to examine the bioaccumulation of trace metals (including Cd) in marine organisms around Qatar and the

region. In 1991, results showed a significant increase in Cd concentration in Qatari fish and other marine organisms (44). El Sayed M & Dorgham M., conducted a study on different species of Macroalgae from the Qatari Coastal Water and they found a high concentration of iron and Cd in some species (45). Recent study aimed to detect the concentration of heavy metals in soft tissue of oyster (*Saccostrea cucullata*) collected from the Lengeh Port coast, Persian Gulf and Iran. Results showed that soft tissue of oyster (*Saccostrea cucullata*) accumulates high Cd concentration, which exceed the permissible limit for human consumption (46).

The gastrointestinal (GI) absorption of cadmium was estimated to be around 5% of the ingested amount, where the daily excretion rate reached 0.005% of the body burden (10). Body health status could affect the absorption rate of Cd. In iron-deficient people, the percentage of absorbed Cd can reach 15-20% (40). Cd interferes with iron absorption leading to anemia. In this case; duodenal iron transporter were suggested to be up regulated and increase Cd absorption in the intestine (47). This evidence is highly correlated to what was reported by Donpunha et al., they found that there is no interference with iron absorption when the Cd-fed mice is supplemented with iron (48). Supplementation with iron and other nutritious elements is significant way to reduce Cd toxicity.

Once Cd is absorbed and enters the circulation it binds to Metallothioneins (MT), which acts as the body's defensive mechanism to reduce Cd toxicity as mentioned earlier. Due to its low excretion rate, Cd circulates in the blood binds to MT and accumulates mainly in liver, kidneys and testes due to their high ability to produce this protein (47).

Indeed, MT is overexpressed in Cd-induced liver toxicity at mice model (49).

Cadmium and Health Effects

Several acute and chronic health effects result from Cd intake based on the consumption rate. Low Cd chronic exposure seems to be one of the great challenges of the 21st century due to its long term health effects (50). After long period of Cd exposure, it accumulates in the body and causes multi-systematic problems. The most affected organs are kidneys and the liver. Cd reaches the kidney in the form of cadmium-metalothionine (Cd-MT). This complex is filtered through the glomerulus, and then reabsorbed by the proximal tubulus after break down of Cd-MT. Cd remains in the tubular cells causing tubular dysfunction and kidney damage. Cd-induced kidney damage is recognized by urinary excretion of low-molecular-weight proteins such as β2-microglobulin, N-acetyl-α-D-glucosaminidase (NAG), and retinol-binding-protein (RBP) (51).

The outbreak of *Itai Itai* disease in Japan in the 1940s illustrates the association between Cd toxicity and skeletal system. In China, similar case was reported with population who lived near to a smelter and used the contaminated river water with the smelter waste discharge for irrigation of their fields (52). People in this situation showed a decrease in the bone density due to low mineralization, increase in osteoporosis and increase in bone fracture rate (14). Similar evidences of increased skeletal fragility and decreased mineral density were reported from animal study in which rats were fed for long-term on dietary Cd at levels corresponding to environmental human exposure (53).

Accumulative evidences suggested that Cd could be a risk factor for cancer. International Agency for Research on Cancer (IARC) classified Cd as human carcinogen (54). Different cancer cases were resulted from Cd exposure such as kidney, lungs, prostate, breast and endometrial cancers in females (55). Cd mimics sex hormones by activating estrogen and androgen receptors and leads to the development of breast and prostate cancer (56). A range of underlying molecular mechanisms has been addressed to initiate cancer. These mechanisms include DNA damage, reactive oxygen species (ROS) production and the consequences of ROS signaling (57).

Body burden result from Cd toxicity might be due to the activation of oxidative stress pathway. Oxidative stress is a response of biological system that disturb the redox balance at the cellular level and result in macromolecules damage such as protein degradation, cross-links in DNA and membrane fatty acid peroxidation (58). Accordingly, Cd has shown to induce oxidative stress and inhibit the antioxidant enzymes (59). Moreover, it induces the formation of free radicals and ROS that has an effective role in tissue damages (60).

Cadmium and Hypertension

In the last few decades, Cd has been considered as a risk factor for CVDs including hypertension. Since that time researchers start to investigate the relationship between Cd and CVDs particularly hypertension. Epidemiological studies revealed the presence of Cd in urine is correlated with the elevation of systolic and diastolic blood pressure (61). In the United States (U.S) general population, Cd has been observed to increase the prevalence of peripheral arterial disease, all participant Cd level was below

the safety standard for Cd (5μg/L) (62). This relationship was also addressed by other epidemiological studies in which Cd corresponded to the prevalence of stroke and heart failure (63). The Korean National Health and Nutrition carried out an Examination Survey for two years (2008–2010) on Korean population over 20 years old. The result of this study showed a significant association between blood Cd and hypertension in women and men (64). Urinary and serum level of Cd has been associated with cardiovascular and cerebrovascular disease (65). A study was conducted on peoples with high urinary Cd levels (≥ 5 μg/g creatinine) and followed up for chronic Cd toxicity such as hypertension. Results showed urine Cd level is attributed with elevation in blood pressure (66). Urine Cd level showed to be higher in hypertensive cats compared to normotensive ones in a cross-sectional study (67). Recently, a demonstration from population study showed an association between blood and urine Cd level with the prevalence of peripheral artery disease among 489 participants (68).

Beside epidemiological studies, *In vivo* studies considered as an important field to demonstrate the relationship between Cd toxicity and blood pressure. A large number of animal studies were conducted using different species with different ways of administration to illustrate this phenomenon. The first study was published by Schroder addressed in 1962 and showed that chronic exposure of low Cd level induce hypertension in rats (69). Similar findings were also addressed by Perry and Erlanger suggested that non-toxic pressor doses of Cd (0.1 to 5 ppm) are able to modulate blood pressure (70). Cd produced hypertension in hypertension sensitive rats when they receive 1-5 mg Cd/L via drinking water, and it was suggested that genetic differences influence Cd-induced

hypertension (71). Significant increase in blood pressure were observed in female Long-Evans rats exposed to 0.1, 0.25, or 0.5 ppm of Cd via drinking water for 6 to 18 months (72). Albino rats received 15µg/ml Cd for 30 days showed an obvious increment in blood pressure, where systolic and diastolic blood pressures rose from 102.8 +/- 7.0 and 81.2 +/- 3.8 mm Hg to 128.1 +/- 4.6 and 107.9 +/- 7.4 mm Hg (73). Recently, evidence was reported that Cd modulates hypertension. A study conducted by Donpunha et al. showed a significant increase in the systolic, diastolic blood pressure and the mean arterial blood pressure of mice treated with 100 mg/l $CdCl_2$ via drinking water for 8 weeks (24). These evidence support that Cd could be a risk factor for hypertension.

Blood vessel is a hollow tube that consist of two main layers, the endothelial cells (ECs) which are a line of cells that cover the entire blood vessels and form as a connection bed between blood and the body tissues (74). This connection ensures the delivery of oxygen and nutrients to the cells for survival. Beneath the ECs is a thick layer of VSMCs. VSMCs is the muscular part of the vessel that is responsible for its contraction. Once Cd enters human body, it reaches the blood stream and targets the vascular wall (47). Changes in the ECs, vascular receptors expression and contractility level are well-known factors to elevate blood pressure. Evidence suggested that Cd could target blood vessels and result in blood pressure modulation. Cd can potentiate cytotoxicity of the cells in the vessel wall. Cd showed a cytotoxic effects to ECs by destroying the monolayer of ECs (75). These finding could be related to the effect of Cd on VE-cadherin (cell adhesion molecules) that has a major role in connecting the cells together as reported by Prozialeck et al. (76). They found that 10 and 100nM cadmium

decrease the cell contacts through the monolayer of HUVEC cells. Disruption of ECs contacts leads to the alteration in the structure of vascular endothelium. Other process that might affect endothelial integrity is the induction of cell death. It has been shown that ECs are targeted by Cd and end with cells death (77). It has been shown that Cd induce cell apoptosis through the p38 MAPK dependent mechanism in brain microvascular ECs (78). Cd-induced-endothelial cell death revealed to be due DNA strand damage and p53 downstream activation (79). Cd showed to inhibit the migration, proliferation and tube formation of HUVECs when they exposed to 1.0 μM-1.0 mM of $CdCl_2$ (80). Similar findings were revealed recently and suggested that Cd inhibit the endothelial migration and tube formation at dose 5μM of $CdCl_2$ (81). These processes are key players and have an important role in angiogenesis. Consequently, Cd has a direct effect on angiogenesis and tube formation, but this effect showed to be dual effect. Vascular endothelial growth factor (VEGF)-dependent mechanism of angiogenesis was tested on ECs (HUVECs) treated with different concentration of $CdCl_2$ (2.5-40 μM). Low Cd concentration showed to increase the secretion of VEGF and induce the tube formation in HUVECs. Unlike low concentration, high Cd concentration showed an adverse effect and inhibits the tube formation (82). Therefore, any damage in the endothelium will alter its permeability (77), and result in the formation of gaps between the cells. Gaps formed between the endothelial cells, helps Cd to transfer from blood stream to the medial layer (76).

When Cd damages the unique layer of ECs it reaches the VSMCs, which works as a recipient for Cd toxicity. Kaji T et al. found that vascular smooth muscle cells exhibited high sensitivity to Cd toxicity compared to Chang-liver cells. High sensitivity of VSMCs

(without any species-related differences) suggested to be due to accumulation of such metal inside the cells (83). Consistent with these findings, Cd showed to accumulates in the medial layer of smokers aorta compared to intima and adventitia (84). Cd could affect the viability of VSMCs and lead to cellular dysfunction. VSMCs viability decreased in a concentration dependent-manner (85). In the same study, $CdCl_2$ also showed to increase ERK 1 & 2 in a biphasic manner. This increment reached the maximum when Spontaneously Hypertensive Rat (SHR) VSMCs exposed to 1 and 4 μM. On the other hand, a reduction was revealed with Wistar Kyoto rat (WKY) cells at 2μM. It has been indicated also that $CdCl_2$ elevated PKC expression in both cell types, where the highest elevation was observed in SHR VSMCs (85). Moreover, adverse effects may initiated due to VSMCs Cd toxicity, Mikhaleva et al. investigated that chronic oral administration of 2.5 mg/100 g body weight $CdCl_2$ to albino rat might cause hypertrophy in the left ventricular cardiomocytes as well as arterial walls (86). Interestingly, more complications could occur due to endothelial dysfunction that trigger different factors associate in the pathology of the vascular wall. These findings are supported by other study, Park SL et al reported that Cd induces the expression of adhesion molecules such as (ICAM-1) and (VCAM-1) which facilitate the migration of immune cells through the vessel wall (87). Infiltration of immune cells helps them to reach the intimal layer and start to secrete inflammatory signals as a sign of inflammatory response to injury. Consentient with these findings, leukocytes number was significantly increase in Cd-treated mice causing a systematic inflammation as consequences of Cd toxicity (88). An interesting review done by Olszowski et al. suggested that micro molar concentration of Cd result in up-

regulation of inflammatory mediators in different studies (*in vivo* and *in vitro*). Increase in IL-6 was revealed in human fibroblast and other cell lines treated with Cd (89). Moreover, TNF-α level in plasma was also increased in response to Cd administration in rats (90). IL-6, TNF-α and other cytokines have a critical role in disrupting the vascular wall (91). These evidences could be linked directly to the effect of Cd on the secretion of inflammatory mediators and induce inflammatory response.

Inflammatory response stimulates VSMCs migration and proliferation to contribute in the formation of atheroma in the intima. It has been suggested that low level of Cd (100nM or less) may enhance the proliferation of vascular smooth muscle cells through intracellular calcium-dependent signaling pathway (92). Migration could enhance by the matrix metalloproteinases (MMPs). MMPs facilitate migration by catalyze and removal of the basement membrane that surround VSMCs (34). It has been shown that MMPs are elevated in rat treated with Cd as reported by Kirschvink et al. (93). In addition, findings were summarized by Newby A. C. suggested that MMPs activation increase and induce VSMCs migration (34). Based on these data, VSMCs could be migrated due to Cd toxicity as a key element in vascular pathogenesis.

Cd could also affect the vascular wall by modulating the molecular pathways that control the blood pressure. For example; Nitric oxide (NO) is a signaling molecule that regulates high blood pressure and mediates vascular homeostasis (94). NO is synthesized by NO synthase (NOS) proteins. NOS are constitutively expressed such as neuronal NOS (nNOS) and endothelial NOS (eNOS). Other NOS form can be regulated by the stimulation of cytokines and called inducible NOS (iNOS). The reduction in NO

production mediates blood pressure and cause hypertension. This reduction can be achieved by modulating the NO bioavailability or through NOS expression. A reduction in eNOS protein level was observed in rat treated with Cd at doses 10 and 50 ppm (95). Other mechanism was addressed by Majumder et al. in which Cd inhibits eNOS phosphorylation (96). In contrast, conflicting data were published by Takahashi et al., which revealed that *in vitro* Cd treatment of aortic strips significantly up-regulated the expression of iNOS and eNOS (97). Overexpression of eNOS was also showed recently in rats intoxicated with low chronic $CdCl_2$ (100mg/L), accompanied with a decrease in NO bioavailability (50). Moreover, NO bioavailability is another angle that can be addressed as pathway of Cd-induced hypertension. NO formation was decreased in rat received acute i.c.v (intracerebroventricular) Cd injection (98). Administration of 50 or 200 ppm $CdCl_2$ via drinking water showed a decrease in serum NO concentration (99). This could be liked to the initiation of oxidative stress, one of the main endogenous actions that triggered by Cd toxicity as mentioned earlier. Vascular oxidative stress indicated a decrease in NO production (100). Therefore, any alteration in the NO production affects blood pressure.

Studies were applied to investigate the activity of the vascular wall in response to several agonist and antagonist that has a role in initiating the vascular tone. Blood vessel contractility is initiated by several mechanisms one of them are EP or NE hormones. These hormones are known to target adrenergic receptor expressed in VSMCs to induce contraction. The concentration of these hormones is regulated by Catechol-*O*-

methyltransferase enzyme (COMT). This enzyme showed to be inactivated due to Cd toxicity (101).

Vasoconstriction responsiveness to Cd on isolated blood vessels was investigated in order to clarify the relationship between Cd and hypertension. Cd showed to have several actions on the neurohumoral mechanisms that mediate the vasculature function (102). In 1978, Nechay et al. found that Cd can act differently on the vascular bed based on the exposure level. High pressor response to NE was observed when rat injected with low Cd dose (1-2.25mg/kg) compared to high dose (7.5 mg/kg) (103). Cd showed a dual effect on isolated rat thoracic aorta treated with a serial concentration of Cd (10^{-9} – 10^{-4} g/ml). Low doses showed to induce vessel contraction while the high doses induce relaxation (104). The sensitivity of the vascular bed to NE and the perfusion pressure were elevated in $CdCl_2$-induced hypertensive rats (105). Similarly, exogenous NE potentiate the vasoconstriction of mesenteric artery isolated from rat received cadmium chloride intragastrically (20 mg/kg body weight/week), where the 50% effective doses (ED_{50}) showed to be lower in Cd-poisoned rats (106). These results could be linked to the increase in the sensitivity of postsynaptic alpha-adrenergic receptor to NE. In response to phenylephrine and acetylcholine, vasoconstriction and vasodilation were augmented when aortic strips were incubated with 10μM $CdCl_2$ (97). The contractile response to phenylephrine (alpha 1 AR agonist) was increased in the aorta of Cd-treated rat (100mg/L via drinking water) (50). In term of vascular relaxation, Cd showed to suppress vascular muscarinic receptor sensitivity to acetylcholine in isolated rat aorta. The sensitivity was significantly decreased at 10 and 50 ppm Cd exposure (25). Gokalp et al

2009 noticed that relaxation responses were reduced in thoracic aorta of Cd-hypertensive rat (intraperitoneal administration 1mg/kg/day for 15 days), and they suggested that, this observation may be related to the inhibition of NO production and endothelial damage (107).

Hypothesis and study objectives

Vasoconstriction of the blood vessels has a critical role in evoking blood pressure. Several mechanisms were investigated, but still few available data showing how Cd-induce hypertension. Among all hypertension pathways, in this study we focused on the alpha 1 adrenergic receptor pathway because it has been poorly investigated. From the available evidence, we found that Cd inhibits the function of Catechol-*O*-methyltransferase enzyme (COMT) which regulates epinephrine and norepinephrine. Consequently, the vascular reactivity showed to be augmented in response to norepinephrine and phenylephrine as an agonist of α_1 AR. Based on these previous findings and what is known to us, the direct effect of Cd on the α_1 AR receptor is poorly investigated. This led us to hypothesize that **cadmium increases expression and/or activation of vascular α_1 AR**. Moreover, the literature showed that Cd has an effect on the on the function and morphology of vascular wall cells, which leads to vascular dysfunction. Little is known about the effect of Cd on VSMCs phenotypic modulation as an indirect mechanism of blood pressure elevation. Thus, another hypothesis was stated that **cadmium induces the phenotypic modulation of vascular smooth muscle cells.**

In order to fill the gaps in literature and to dissect new role on how Cd-induce hypertension, we aimed in this study to:

1) Determine the effect of cadmium on the expression and activation of vascular α1 adrenergic receptors.
2) Determine the effect of cadmium on VSMCs phenotype.

CHAPTER 2: RESEARCH METHODOLOGY

1) Cell Culture:

Human aortic vascular smooth muscle cells (HASMCs) (Cell Applications INC; U.S), were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco; U.S; cat# 31885049) mixed equally (50:50) with F-12 media (Gibco; U.S; cat# 21765037). DMED/F-12 mixture was supplemented with 10% FBS, 1% Glutamine and 1% Antibiotic-Antimycotic cocktail (Gibco; U.S; cat# 15240062). Cells were incubated at 37°C in a humidified chamber in an atmosphere of 5% CO_2. Cells were used at passages between 9 and 12. Before treatment, cells were made quiescent by serum starvation (0.5% FBS-containing media) for 24 hours.

2) Western Blotting:

Cells were harvested into a lysis buffer (60mM Tris pH 6.8 and 2% SDS). Cells were scraped, sonicated for 5 to 10 seconds and then centrifuged at 10,000g for 15 min at 4°C. The supernatant was then quantified for protein concentration (BCA, Thermo Scientific, U.S , Cat#23227) and stored at -80°C.

Equal protein samples were mixed with 5X sample buffer (4% sodium dodecyl sulfate (SDS), 20% glycerol, 0.5M Tris pH 6.8, 10% β-Mercaptoethanol and 0.10% Bromphenol blue) and loaded onto 10% SDS-polyacrylamide gel. Then the migrated proteins were transferred to a PVDF membrane at 100V for 1 hour. The membrane was washed 2 times for 5minutes each with Tris-buffered saline and Tween20 (TBST)

(0.02M Tris, 0.15M NaCl and 0.1% Tween) and blocked in 5% non-fat milk in TBST. The blocking was performed for overnight at 4°C on rocking plate. In the next day the membrane was washed three times with TBST for 5 min each. After that, the membrane was incubated with rabbit monoclonal primary antibody against α_1 AR (1:1000 dilution in TBST) (Abcam; UK; Cat# ab137123) for 1hr. After incubation, the membrane was washed 3times for 10mins each. Then membrane was incubated with Goat polyclonal secondary antibody (abcam; UK; Cat# ab97051) 1:5000 dilution in 5% non-fat milk-TBST for 1hr. After that, the membrane was washed 3times/15mins. Bands were detected using enhanced chemiluminescence and quantified. Three independent experiments were performed for each treatment.

The same membranes were stripped following harsh stripping protocol from abcam*. Reprobing was done by applying Glyceraldehyde 3-Phosphate Dehydrogenase antibody (GAPDH) as a loading control. GAPDH bands were used for Western blot normalization.

**Harsh stripping:*

Stripping buffer (20 ml SDS 10%, 12.5 ml Tris HCl pH 6.8 0.5M, 67.5 ml ultra pure water, 0.8 ml β-mercaptoethanol) was warmed to 50°C. Membranes were covered with enough stripping buffer in a tight plastic box (j50°C up to 45 min with some agitation). After disposing of the stripping buffer, the membrane was placed under running water for 1 hr. Then it was washed extensively with TBST for 5min to remove β-mercaptoethanol traces. After following all these steps, the membrane is blocked and the above western protocol was continued.

3) Migration Assays:

Chemotaxis chamber:

24-multiwell insert system (8 um pore size, polyethylene terephthalate (PET) membrane) was used for this experiment. 10,000 cells were seeded with serum free media in the insert of the chamber. Complete media (10% FBS, 1% Glutamine and 1% Antibiotic/Antimycotic cocktail) was added to the lower chamber to serve as chemotactic factor. Treatment was applied directly after seeding: control, 10 nM, 1000 nM and left for 24 hrs at 37°C in a humidified chamber with an atmosphere of 5% CO_2.. 24 hrs. post-treatment, media was aspirated and cells were washed 2 times with PBS. Fixation solution (4% formaldehyde in PBS) was added for 10 min. The fixative was then aspirated and the inserts washed 2 times with PBS, incubated with 100% methanol for 20 min, then washed 2 times with PBS. Staining (1% crystal violet) was applied for 5 min followed by washing with PBS. Non-migrated cells were removed by sterile cotton swap. Photos were taken from at least 5 random fields by an inverted contrast phase microscope (IX71 Olympus) to count the number of migrated cells.

Wound healing:

40,000 VSMCs cells were seeded in 6-well culture plate and left till reached confluence. They were then serum-starved for 24 hrs. A scratch/wound was created in the center of monolayer by using 200µl tip. Treatment (10 and 1000nM) was added directly after wound creation. Wound healing was observed and photomicrographs were taken every 2 hrs.

4) Senescence-associated beta-galactosidase (SA-gal) activity:

Cells were seeded in 6-well plate until they become sub-confluent and serum-starved for 24 hrs. Treatment was applied for 48 hrs as the following: control, 10 nM and 1000 nM. 48 hrs post-treatment, cells were washed twice with PBS. Fixation solution was added (2% formaldehyde and 0.2% glutaraldehyde in PBS buffer) and incubated at room temperature for 5 min. fixation solution was removed and cells were washed twice with PBS. Staining was performed (40 mM citric acid/Na phosphate buffer, 5 mM $K_4[Fe(CN)_6]$ $3H_2O$, 5 mM $K_3[Fe(CN)_6]$, 150 mM sodium chloride, 2mM magnesium chloride and 1 mg/ml X-gal) and cells were incubated for 16 hrs at 37°C incubator. By the end of incubation period, staining solution was removed and cells were washed twice with PBS and once with methanol, plate was left for air dry. Photomicrographs were taken from random fields and positive β-gal cells were counted and represented senescent cells.

5) VSMC Hypertrophy:

8000 VSMCs were seeded in 6-well plate and the next day, they were serum-starved for 24 hrs. Cells were treated with $CdCl_2$ as the following: control, 10 and 1000 nM. Photos were taken for each treatment after 48. Cell areas were measured by using xyz software (IX71 Olympus).

6) VSMC Viability:

300 VSMCs cells were seeded in 96-well plate to reach 20-30% confluence in the next day. The plate was designed to study the concentration and time effect. When the

cells reached 20-30% media was changed to quiescent for 24 hrs. A wide range of $CdCl_2$ concentration was used: control, 10, 50, 100, 500, 1000 and 2000 nM. Each treatment has three replicates. The time duration was 24, 48 and 72 hrs. Then cells viability was determined using CellTiter-Glo Luminescent Assay (Promega; U.S) and following manufacturer protocol. First, plate was equilibrated at room temperature for 30 min. Second, 50 μl of CellTiter-Glo® Reagent (CellTiter-Glo® Buffer/Substrate mixture supplied with the kit) was added to each well, and the contents were mixed for 2 min on an orbital shaker to induce cell lysis. Then plate was incubated at room temperature for 10 min to stabilize the luminescent signal. Finally, luminescence was recorded by using Luminometer (Promega; U.S).

7) *In vivo*: Wistar Rat

Male Wistar rat (N=40) were purchased from Charles River (UK) weighing 200-250g. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by Qatar University Institutional Animal Care and Use Committee (QU-IACUC 010/2013). Rats were housed in a controlled lab conditions with a room temperature (18-25ºC), humidity (30-70%) and light cycle (12:12 h light-dark). Rats were grouped into two groups. Control group (N=15) received filtered tap water as drinking water, whereas the Cd-treated group (N=25) were received filtered tap water containing $CdCl_2$ (15mg/kg of body weight/day) continuously for eight weeks and fed with standard animal diet. All animals have free access to food and water. Food consumption, water intake and body weight gain were measured weekly.

8) Blood Pressure Measurements:

Systolic (SBP), Diastolic Blood Pressure (DBP), Heart Rate (HR) and Mean Arterial Pressure (MAP) were measured using the CODA tail-cuff blood pressure system (Kent scientific Corporation; USA). Rats were picked randomly restrained in the rat holder and placed in a worming pad. The occlusion cuff placed close to the base of the tail, while the Volume Pressure Recording (VPR) cuff placed 2mm away from the occlusion cuff. The rat allowed to be thermoregulated. The temperature was recorded by infrared thermometer, which should be between 32-35°C. Five cycles were set as an acclimation cycles and fifteen as a regular cycles.

9) Mesenteric arteries isolation:

Rats were placed in CO_2 chamber and euthanized by gradual release of CO_2 from compressed CO_2 cylinder. Immediately after euthanasia the rat was dissected and the mesentery was isolated and placed directly in a cold Physiological Salt Solution (PSS) (112mM NaCl, 5mM KCl,1.8mM $CaCl_2$, 1mM $MgCl_2$, 25mM $NaHCO_3$, 0.5mM KH_2PO_4, 0.5mM NaH_2PO_4, 10mM Glucose). Under stereomicroscope (Olympus SZ61). The small mesenteric arteries were isolated, cleaned from fats, and placed in cold PSS solution.

10) Vascular Reactivity:

Two segments of small mesenteric arteries (one from control and one from treated) were mounted on two separate mounting jaws in one chamber of an isometric myograph (model 510A, DMT, Denmark) containing 10 ml of PSS solution. The vessels

were maintained at 37°C and continuously aerated with a mixture of 95% O_2+ 5% CO_2. Vessel segments were then normalized by pre-tensioned to an equivalent of 100 mmHg using an in-built automatic normalization procedure. Normalization procedure is important step to determine the inner diameter of each artery. Vessels were equilibrated for a period of one hour between the mounting and experiment. During this period, 90mM of KCl (once) and 10 μM phenylephrine (twice) were applied to test for viability and optimize the vessels for the experiment. In order to test the endothelial function, acetylcholine (10 μM) was applied after the second phenylephrine to relax the vessel.

Once the equilibration period was done, a dose response test was performed for a serial concentration of phenylephrine (1-30000 nM) followed by a washing period of 30 min. after that, another test was carried out for the norepinephrine (1-30000 nM). Norepinephrine was then repeated in the presence of prazosin ($3x10^{-7}$M), which was applied 30 min before norepinephrine. Prazosin is a selective α_1 AR antagonist.

11) Statistical analysis:

Graphpad prisem software was applied for data analysis. *In vitro* data were analyzed using one-way ANOVA, while *in vivo* data were analyzed using two-way ANOVA. All data were applied to Fisher's LSD test for multi-comparison. Additionally, the 50% effective concentrations (EC_{50}) for each single concentration of agonist was determined using non-linear regression. Data are presented as mean standard error. A value of $P<0.05$ was considered statistically significant.

CHAPTER 3: RESEARCH RESULTS

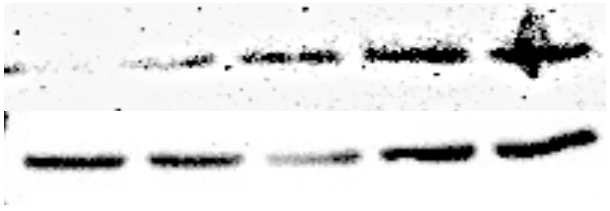
PART I: *IN VITRO* STUDIES

A. Effect of cadmium on the expression of vascular α_1 AR

Cadmium induces the expression of α_1 AR:

To investigate if Cd could evoke α_1 AR expression, Cells were treated with low serial concentration for varying durations. Results showed an increase in the expression of α_1 AR in a concentration (Fig.5) and time (Fig.6) dependent manner. α_1 AR significantly evoked when cells were treated with 500 and 1000nM $CdCl_2$. This increment was 4 and 5 fold-increase for 500 and 1000nM respectively.

(a)



(b)

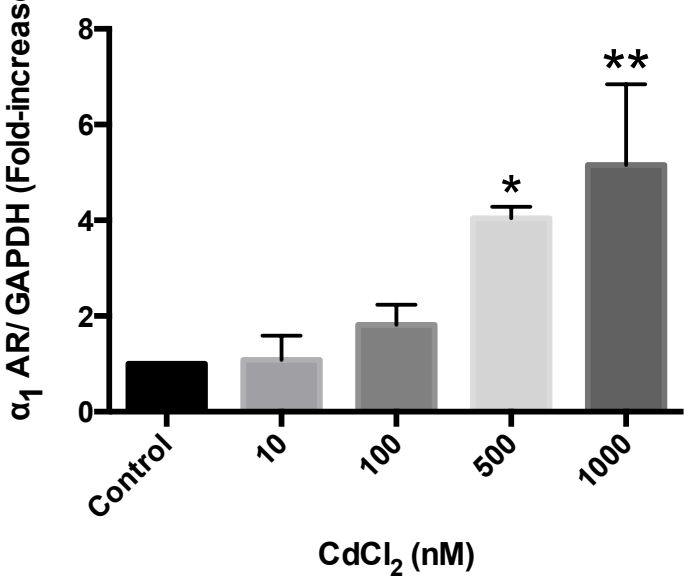


Figure 5: Effect of $CdCl_2$ on α_1 AR expression. HASMCs were treated with a serial concentration of $CdCl_2$: 10, 100, 500 and 1000nM for 48 hrs. (a) Expression of α_1 AR compared to GAPDH. (b) Quantitative measure of α_1 AR normalized with GAPDH. Data are presented as mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$. n=3.

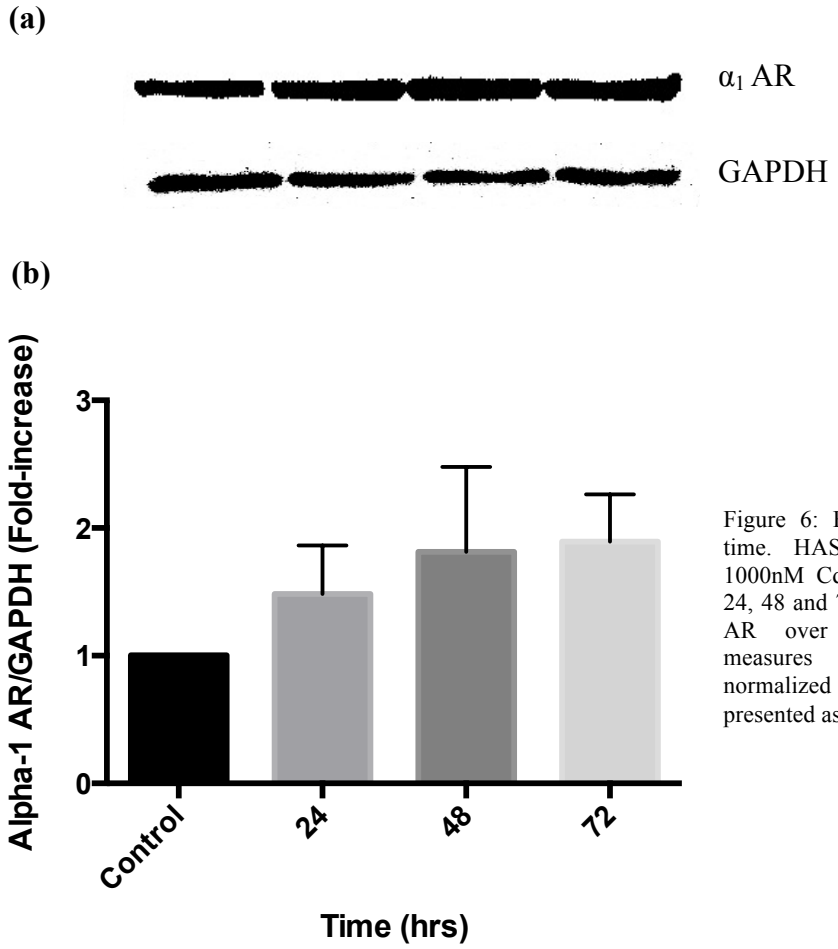


Figure 6: Expression of α_1 AR over time. HASMCs were treated with 1000nM $CdCl_2$ for varying durations: 24, 48 and 72 hrs. (a) Expression of α_1 AR over time. (b) Quantitative measures for α_1 AR expression normalized with GAPDH. Data are presented as mean ± SEM. n=3.

Transcriptional activity and involvement of Protein Kinase A (PKA) pathway:

To determine if the up-regulation of α_1 AR is due to activation of transcription, cells were pretreated with actinomycin D to suppress DNA-dependent RNA synthesis. As previously shown, 1000nM $CdCl_2$ significantly increases α_1 AR expression. Here we show that this expression is abolished by actinomycin D pre-treatment (Fig. 7). To investigate if the expression of α_1 AR is mediated by protein kinase A (PKA) pathway, Cells were pre-incubated with H89, a PKA inhibitor. The expression was abolished with H89 (Fig.8).

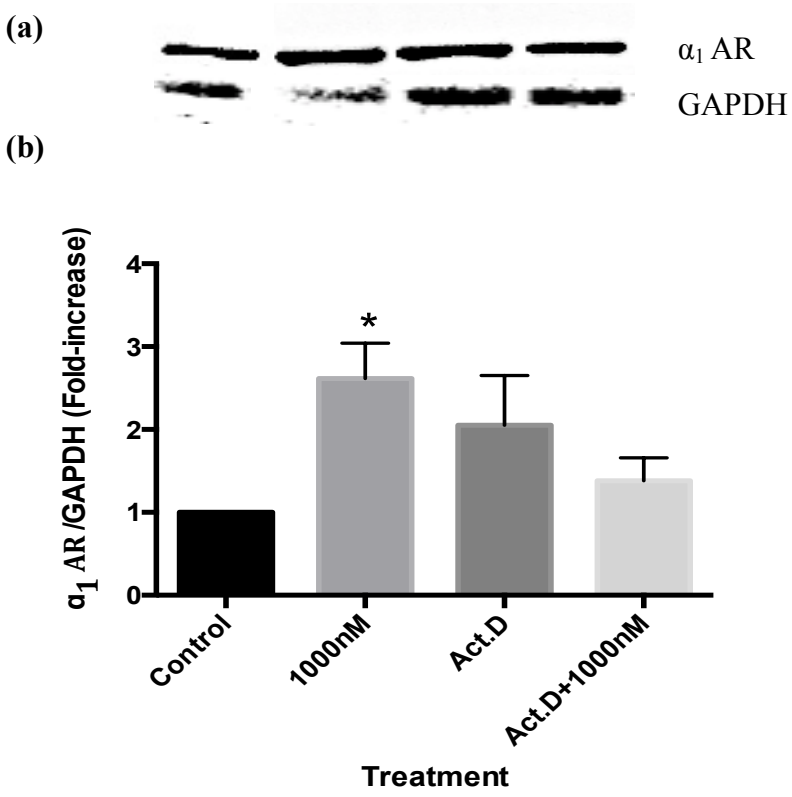


Figure 7: Transcriptional activity of α_1 AR. HASMCs were pretreated with 5μg/ml actinomycin D (DNA-dependent RNA synthesis inhibitor) for 1 hr followed by $CdCl_2$ treatment. Treatment was performed for 48 hrs. Data are presented as mean ± SEM. *Control vs 1000nM. $P < 0.05$. n=3.

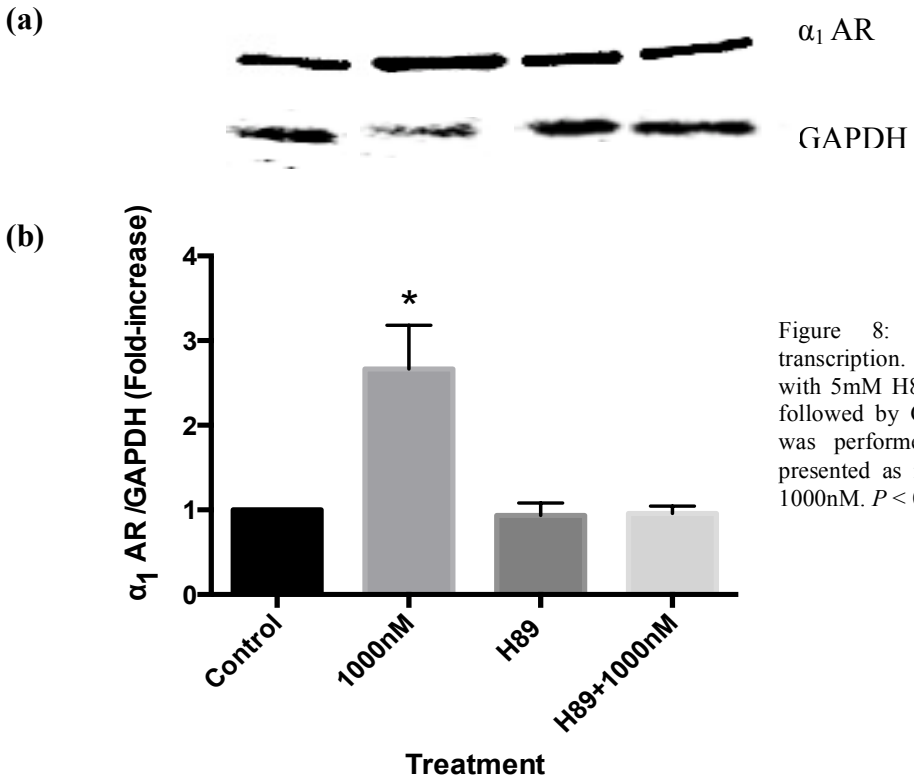


Figure 8: PKA mediate α_1 AR transcription. HASMCs were pretreated with 5mM H89 (PKA inhibitor) for 1 hr followed by $CdCl_2$ treatment. Treatment was performed for 48 hrs. Data are presented as mean ± SEM. *Control vs 1000nM. $P < 0.05$. n=3.

B. Effect of Cadmium on Vascular Smooth Muscle Cells (VSMCs) Phenotypic Modulation

VSMCs Hypertrophy:

To determine the effect of Cd on VSMCs hypertrophy, Cells were treated with 10 and 1000 nM $CdCl_2$ for 48hrs. At 1000 nM cells showed to have a significant increase in cell area compared to control (fig.9).

(a)

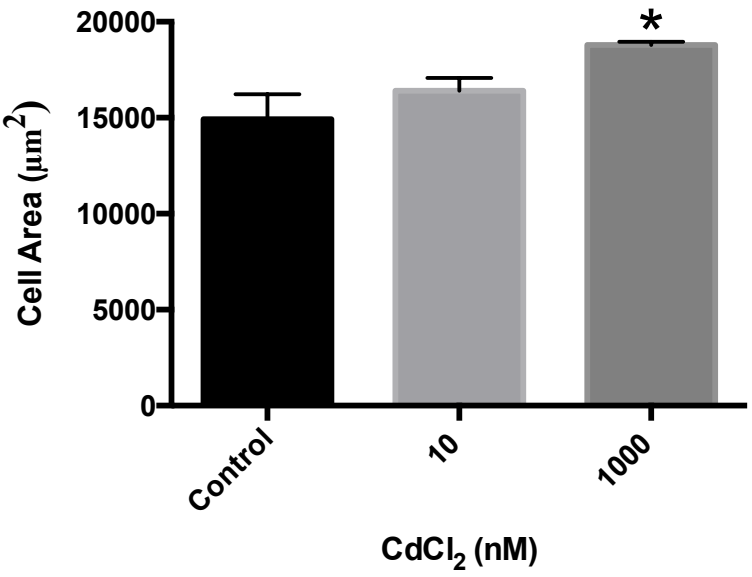
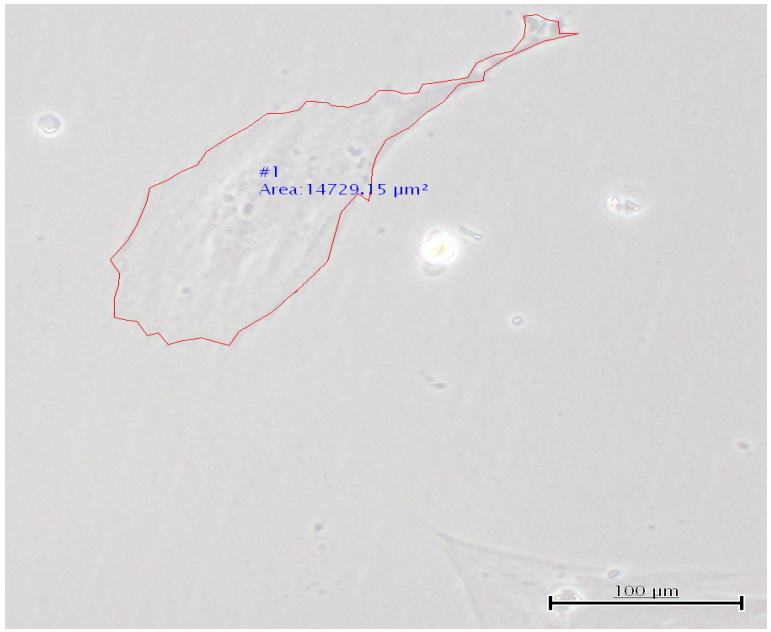


Figure 9: VSMCs hypertrophy. HASMCs were treated with 10 and 1000nM of $CdCl_2$ for 48 hrs and cell area measured using xyz software (a). Control (b), 10nM (c), 1000nM (d).*$P < 0.05$. n=3.

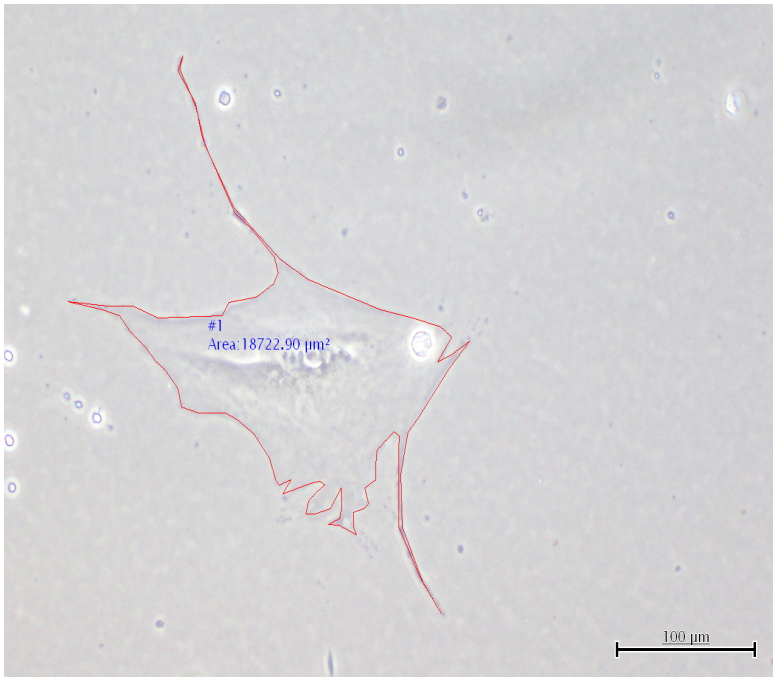
(b)


#1
Area: 14729.15 µm²
100 µm

(c)


#1
Area: 17631.80 µm²
100 µm

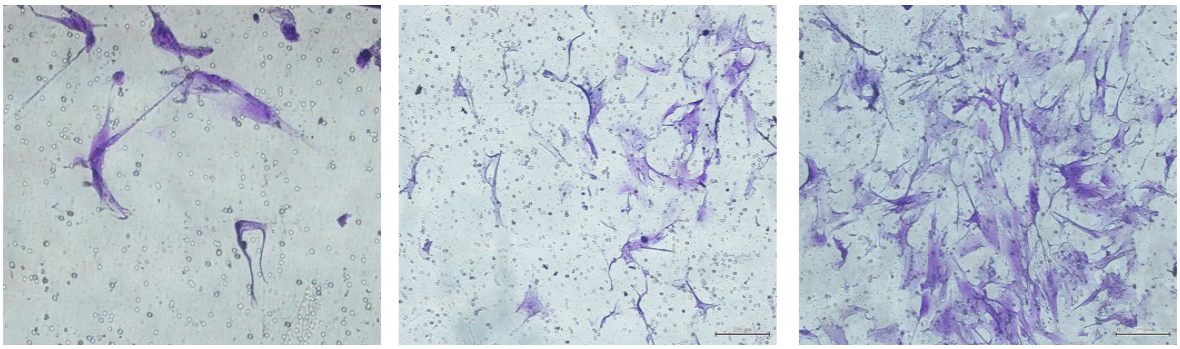
(d)


#1
Area: 18722.90 µm²
100 µm

VSMCs Migration:

To investigate if Cd induces migration of VSMCs. Cells were treated with 10 and 1000 nM $CdCl_2$. Migration chamber assay showed an increase in VSMCs migration in the presence of $CdCl_2$. This increment showed to be significant at 1000nM (fig.10). Wound healing assay showed a faster healing in the presence of $CdCl_2$ compared to control (fig.11).

(a)



Control 10nM 1000nM

(b)

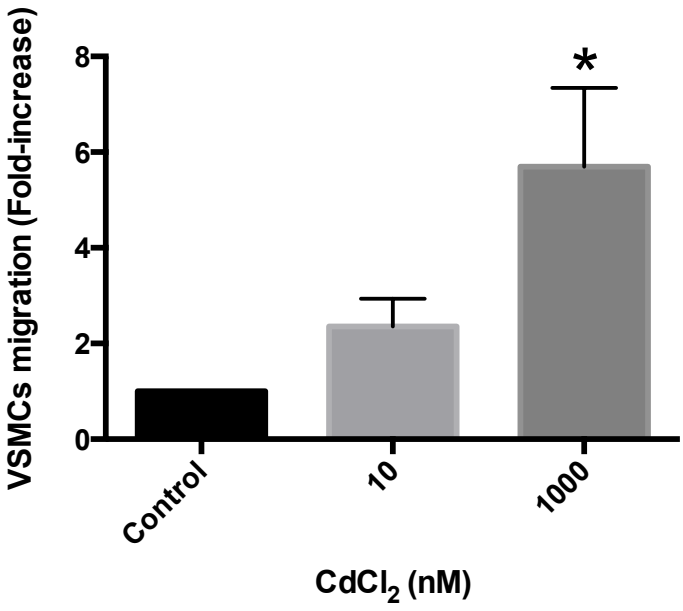


Figure 10: VSMCs migration chamber assay. HASMCs were treated with 10 and 1000nM $CdCl_2$ for 24 hrs. (a) high number of migratory cells stained with crystal violet showed at 1000nM. (b) Migration increased significantly at 1000nM. Data are reported as mean ± SEM *$P < 0.05$. n=3.

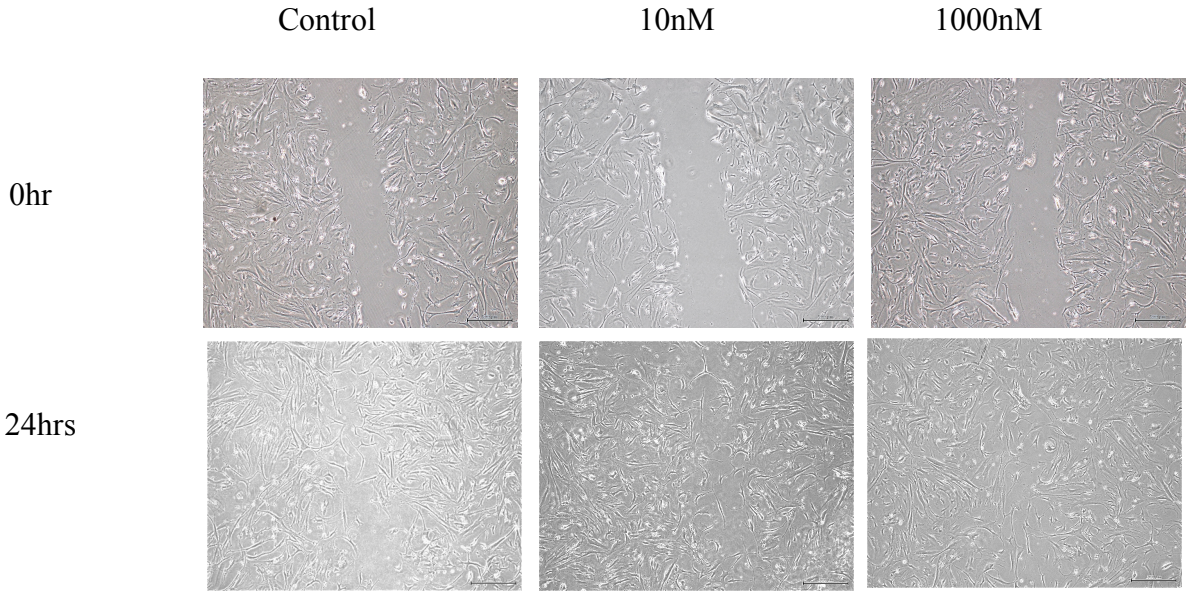
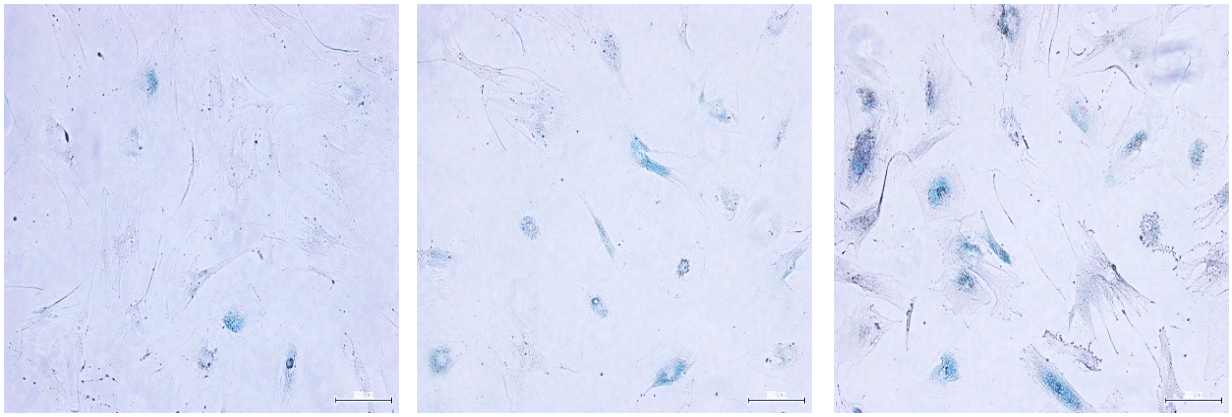


Figure 11: Wound-healing assay. Qualitative results showed faster healing in HASMCs at 24 hrs for the wound created at 0 hrs.

VSMCs Senescence:

To further test the role of Cd in VSMCs phenotypic changes and promote senescence, cells were treated with 10 and 1000 nM $CdCl_2$ for 48 hrs and measured SA-β-gal activity, a senescence marker. Senescence determined by counting SA-β-gal positive cells. Senescence was increased when cells incubated with $CdCl_2$ and showed to be significant at 1000nM compared to control (fig.12).

(a)



Control 10nM 1000nM

(b)

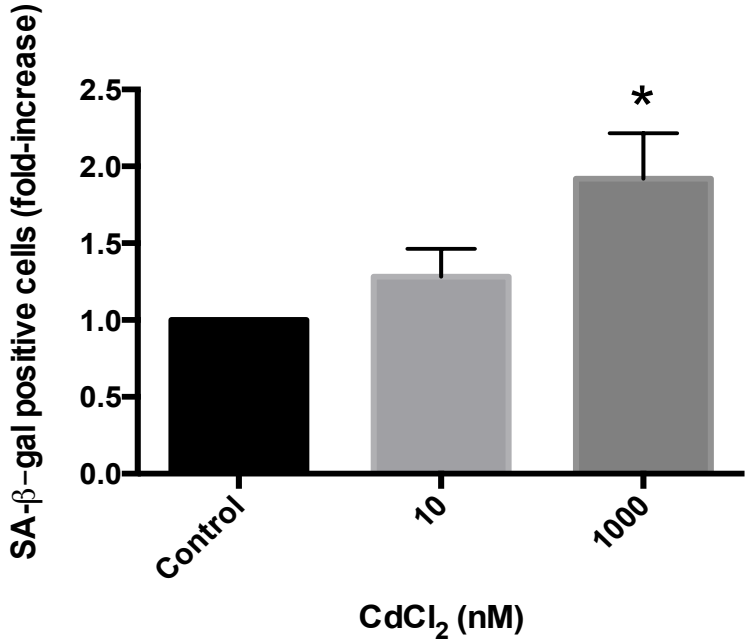


Figure 12: VSMCs Senescence. (a) SA-β-gal positive cells increased with $CdCl_2$ treatment, (b) significant at 1000nM after 48 hrs. Data are reported as mean ± SEM. *$P < 0.05$. n=3.

VSMCs Viability:

In order to detect the effect of Cd toxicity on the viability of VSMCs, 300 cells HASMCs were seeded in 24-well plates to get 20-30% confluent. Cells were treated with a wide range of $CdCl_2$: 0, 10, 50, 100, 500, 1000 and 2000 nM for varying time. No effect was shown in cell viability between control and Cd treatment after 24 and 48 hrs incubation (fig.13, a and b). While after 72 hrs a decrease in viability was observed at 2000 nM (fig.13, c).

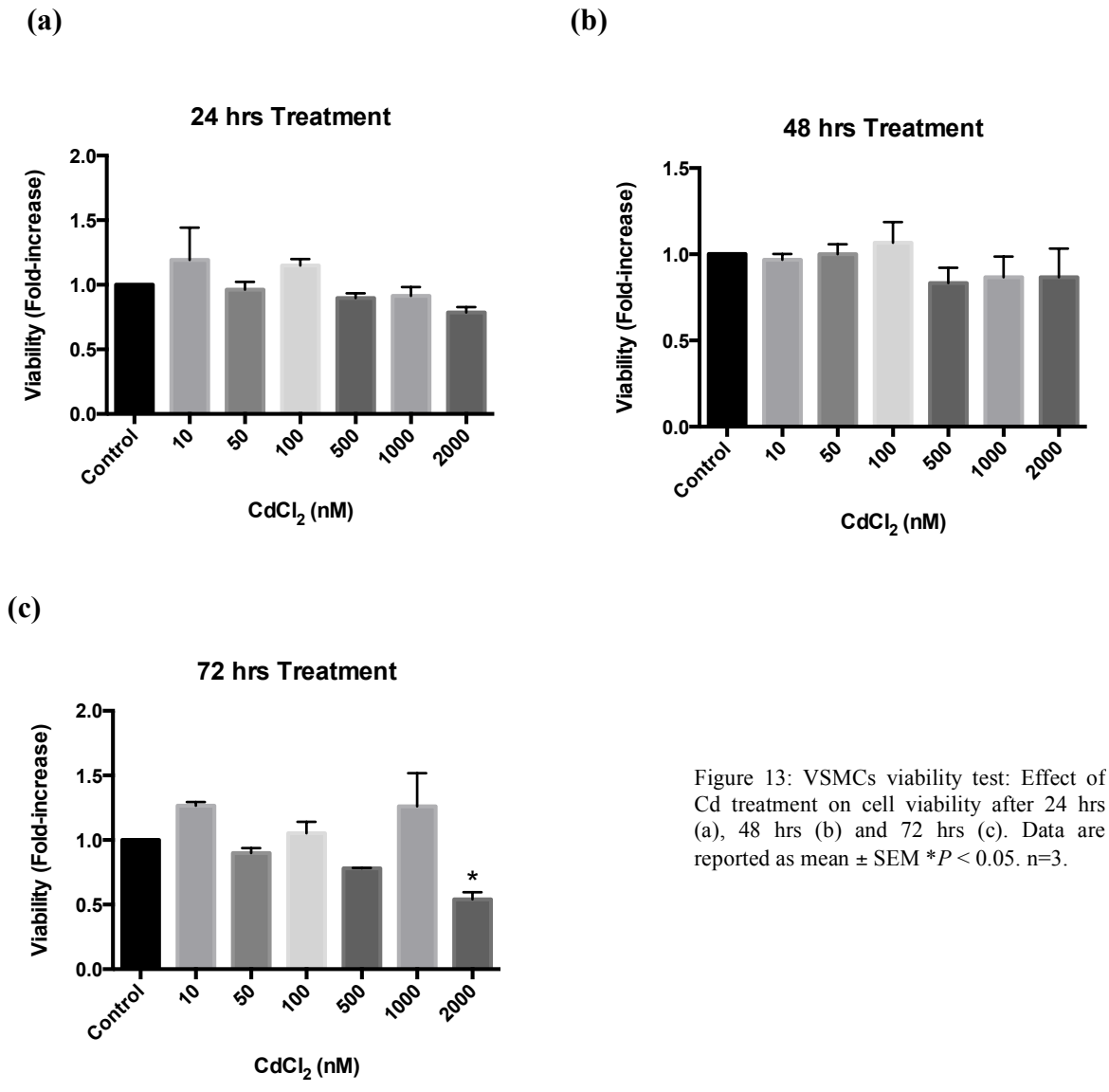


Figure 13: VSMCs viability test: Effect of Cd treatment on cell viability after 24 hrs (a), 48 hrs (b) and 72 hrs (c). Data are reported as mean ± SEM $*P < 0.05$. n=3.

PART I: *IN VIVO* STUDIES

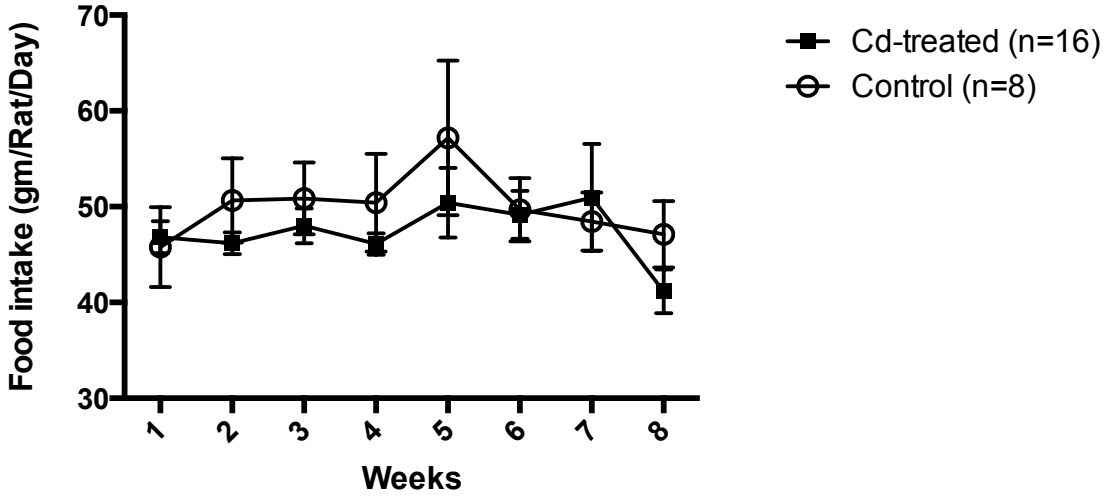
A. Effect of Cadmium on the Sensitivity of Vascular α_1 AR

Food intake and Body weight:

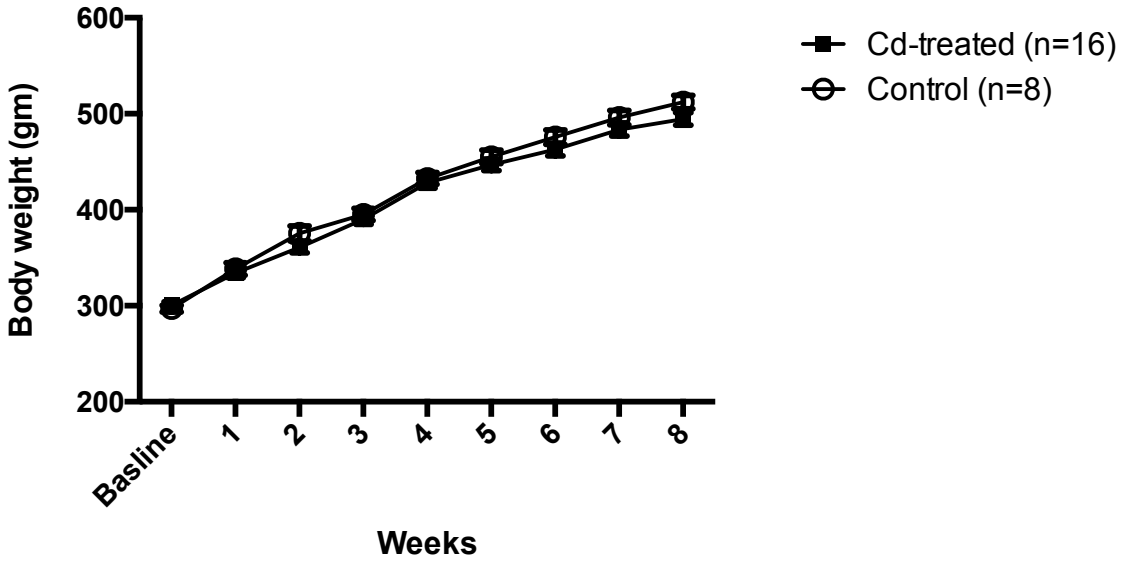
In the present study, the daily food intake was measured weekly during the entire period of the experiment, calculated, and expressed as amount of consumed food in grams/animal/day. The results did not show any significant difference between control and Cd-treated groups (fig.14, a) and (Table.4). The body weight was dramatically increased in both groups during the experiment as a part of normal age related growth, however no significant differences were observed between the two groups (fig.14, b) and (Table.5).

Water Intake: the daily water consumption per animal was measured during the entire experiment and expressed as ingested amount of water in ml/animal/day. The results showed a highly significant decrease in the daily water consumption starting from the first day till the end of the experiment by the Cd-treated group compared to the control group (fig.14, c) and (Table.6).

(a)



(b)



(c)

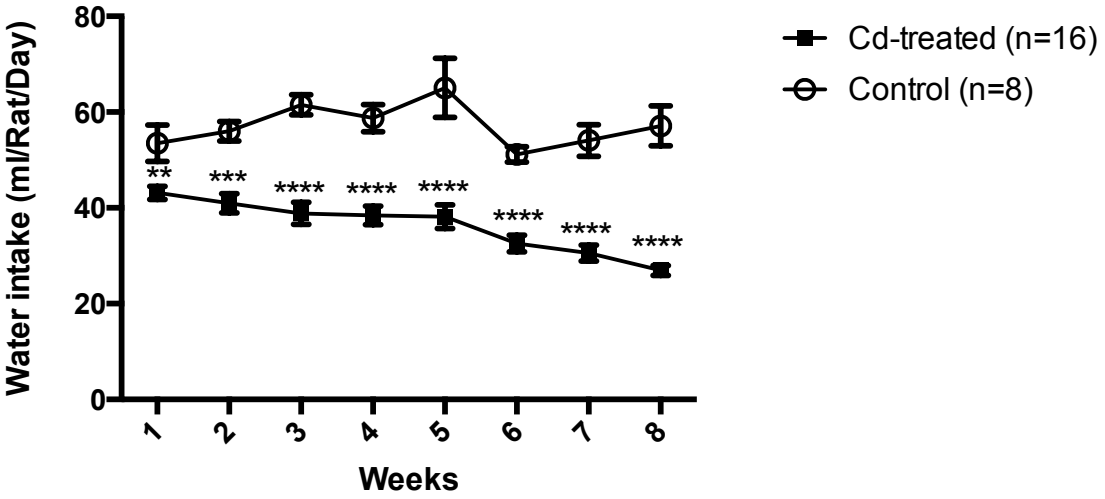


Figure 14: The effects of Cd exposure on: Food intake (a). Body weight (b). Water intake (c). Values are means ± SEM. $^{**}P<0.01$, $^{***}P=0.0001$, $^{****}P<0.0001$.

Table 4: Food intake Means ± SEM (gm/rat/day).

Weeks	Control (n=8)	Cd-Treated (n=16)
Week 1	45.78 ± 4.15	46.81 ± 1.6
Week 2	50.65 ± 4.39	46.18 ± 1.13
Week 3	50.86 ± 3.75	48.00 ± 1.82
Week 4	50.40 ± 5.09	46.10 ± 1.12
Week 5	57.18 ± 8.05	50.42 ± 3.62
Week 6	49.68 ± 3.3	49.16 ± 2.49
Week 7	48.45 ± 3.02	50.96 ± 5.58
Week8	47.11 ± 3.47	41.16 ± 2.28

Table 5: Rats Body weight Means ± SEM (gm).

Weeks	Control (n=8)	Cd-Treated (n=16)
Baseline	297.01 ± 3.63	300.36 ± 3.8
Week 1	338.41 ± 6.5	334.06 ± 4.9
Week 2	375.36 ± 7.6	360.87 ± 5.7
Week 3	395.17 ± 6.4	390.08 ± 5.2
Week 4	432.52 ± 6.1	428.31 ± 5.8
Week 5	455.17 ± 7.1	446.95 ± 5.9
Week 6	475.77 ± 7.5	462.84 ± 6.6
Week 7	496.03 ± 7.5	483.52 ± 6.8
Week 8	512.27 ± 7.04	494.56 ± 6.4

Table 6: Water intake Means ± SEM (ml/rat/day).

Weeks	Control (n=8)	Cd-Treated (n=16)
Week 1	53.48 ± 3.8	43.14 ± 1.38
Week 2	56.02 ± 2.03	40.95 ± 1.98
Week 3	61.53 ± 2.12	38.85 ± 2.3
Week 4	58.73 ± 2.86	38.42 ± 1.92
Week 5	65.04 ± 6.17	38.19 ± 2.45
Week 6	51.17 ± 1.6	32.56 ± 1.71
Week 7	54.07 ± 3.3	30.56 ± 1.69
Week8	57.10 ± 4.16	26.95 ± 1.02

Blood Pressure:

To determine the effect of Cd on blood pressure four parameters were measured immediately before the start of the experiment (baseline) and after 30 days: Systolic Blood Pressure (SBP), Diastolic Blood Pressure (DBP), Heart Rate (HR) and Mean Arterial Blood Pressure (MAP). The results indicated that there was a slight non-significant increase in the SBP baseline value (160.5 ± 3.2) of the control compared to the second group that was assigned for Cd treatment (151.8 ± 2.6) (Table.7). No differences in SBP were observed between control (160.7 ± 4.5) and treated (154.3 ± 3.3) after 30 days of Cd administration (Table.7). Cd did not affect DBP as shown in treated group (108.7 ± 3.15) compared to control (120.3 ± 3.4) (Table.8). Similar results were obtained from MAP (Table.9). Heart rate showed a slight increase in the baseline of control (480.7 ± 15.4) compared to treated (459.3 ± 26.3); yet this change was not significantly different (Table.10). No significant change in the HR was also observed between control (450.4 ± 19.1) and treated (448.04 ± 9.05) 30 days post-treatment.

Table 7: Systolic blood pressure (mmHg) baseline and at 30 days of treatment. Mean ± SEM and % change.

	Control (n=8)	Cd-Treated (n=16)
Baseline	160.5 ± 3.2	151.8 ± 2.6
30 days post-Cd-treatment	160.7 ± 4.5	154.3 ± 3.3
% Change	0.13 %	1.6 %

Table 8: Diastolic blood pressure (mmHg) baseline and at 30 days of treatment. Mean ± SEM and % change.

	Control (n=8)	Treated (n=16)
Baseline	121.5 ± 2.3	109.6 ± 3.16
30 days post-treatment	120.3 ± 3.4	108.7 ± 3.15
% Change	1.04 %	0.84 %

Table 9: Mean arterial blood pressure (mmHg) baseline and at 30 days of treatment. Mean ± SEM and % change.

	Control (n=8)	Treated (n=16)
Baseline	134.2 ± 2.5	123.38 ± 2.9
30 days post-treatment	133.4 ± 3.7	123.59 ± 3.2
% Change	0.61 %	0.16 %

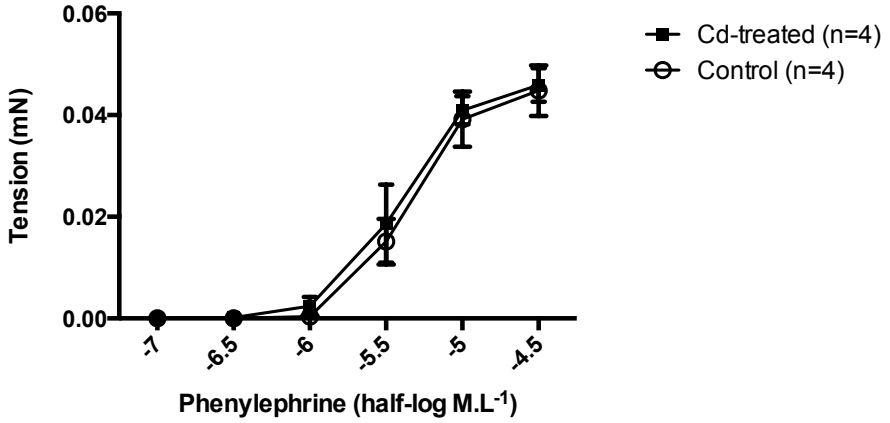
Table 10: Heart rate (bpm) baseline and at 30 days of treatment. Mean ± SEM and % change.

	Control (n=8)	Treated (n=16)
Baseline	480.7 ± 15.4	459.3 ± 26.3
30 days post-treatment	450.4 ± 19.1	448.04 ± 9.05
% Change	6.3 %	2.4 %

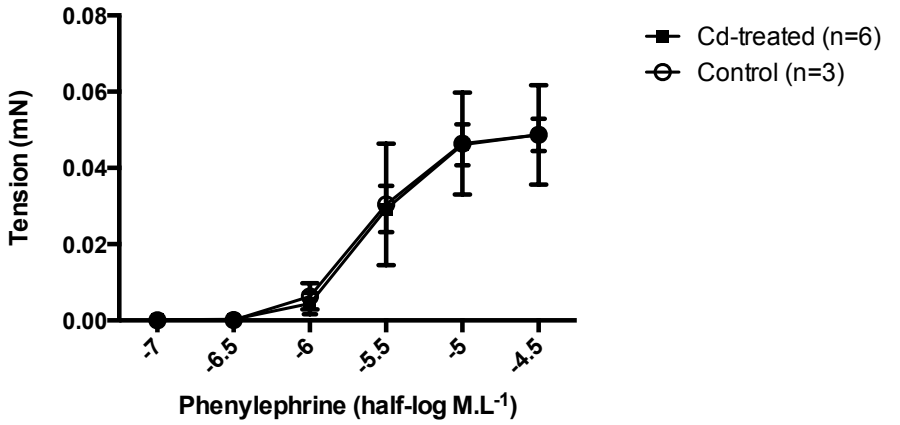
Vascular Reactivity:

The effect of Cd on the contraction of mesenteric arteries in response to NE (ARs agonist) and phenylephrine (selective α_1 AR agonist) was studied to determine the vascular α_1 AR sensitivity. In response to phenylephrine, the results showed that there was a slightly shift to the left for the treated group after 30 days of Cd treatment (-5.442 ± 0.139), which was not significantly differ from the control (-5.358 ± 0.067) (fig.15, a). In contrast, no shift at all was observed after 60 days (fig.15, b). The 50% effective concentrations for both groups were similar (Table.7). By conducting time-dependent comparison between control group and treated group in response to phenylephrine, both control and treated groups 60 days post-treatment revealed an enhancement in the trend, but this enhancement was not statistically different (fig.15, c and d) (EC_{50} values showed in Table.7).

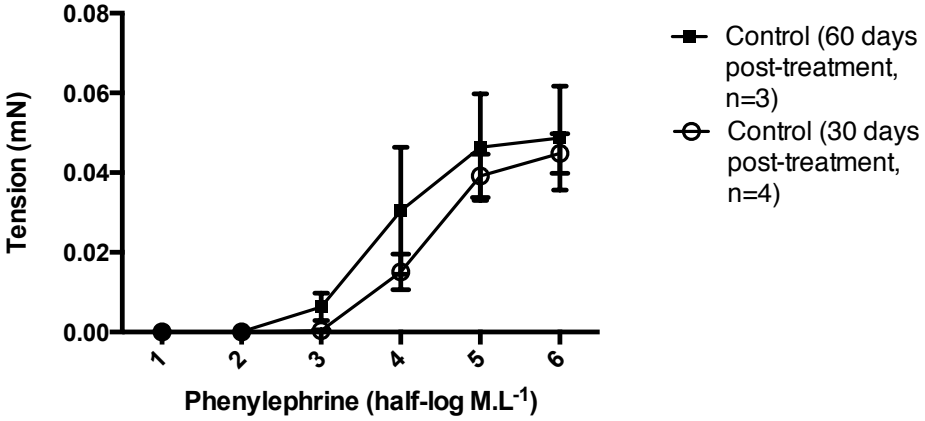
(a)



(b)



(c)



(d)

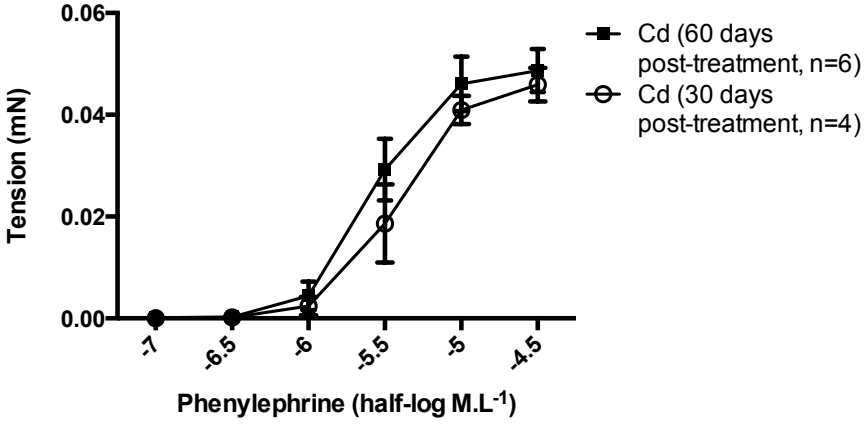


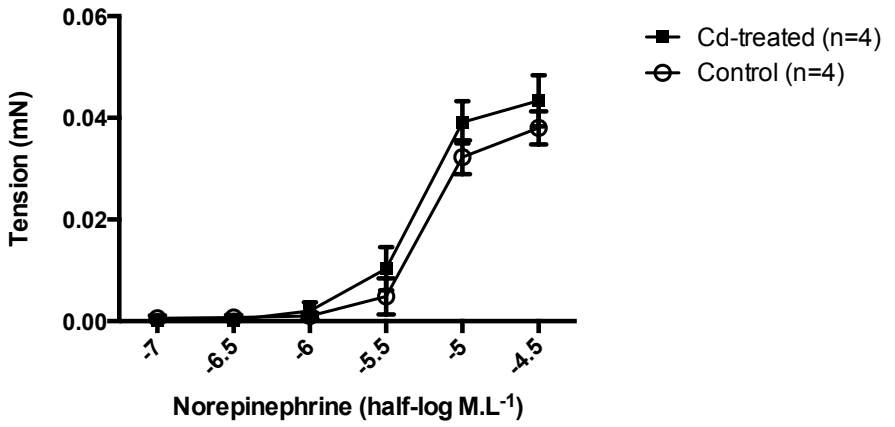
Figure 15: The effect of Cd exposure on concentration response curve to phenylephrine: control vs Cd-treated 30 days post-treatment (a), control vs Cd-treated 60 days post-treatment (b), control 30 days post-treatment vs control 60 days post-treatment (c), Cd-treated 30 days post-treatment vs Cd-treated 60 days post-treatment (d) Values are means ± SEM. $^{**}P<0.01$.

Table 11: The 50% effective concentrations (EC_{50}) for control and Cd-treated in response to phenylephrine, 30 days and 60 days post-treatment.

Duration	Control	Cd-treated
30 days post-treatment	-5.358 ± 0.067	-5.442 ± 0.139
60 days post-treatment	-5.579 ± 0.167	-5.549 ± 0.086

In Cd-treated group, the contractile response induced by NE was increased in rat mesenteric artery (-5.358 ± 0.119) compared to control (-5.224 ± 0.096) 30 days post-treatment, but this increment was not significantly different (fig.16, a). No effect of Cd was shown 60 days post-treatment (EC_{50} values showed in Table.5) (fig.16, b). In time dependent comparison, both curves (control and treated) were augmented in response to NE 60 days post-treatment and showed a significant increase at dose $10^{-5.5}$ $M.L^{-1}$ (fig.16, c and d). The EC_{50} values were -5.673 ± 0.023 and -5.589 ± 0.041 for control and treated respectively 60 days post-treatment (Table.5).

(a)



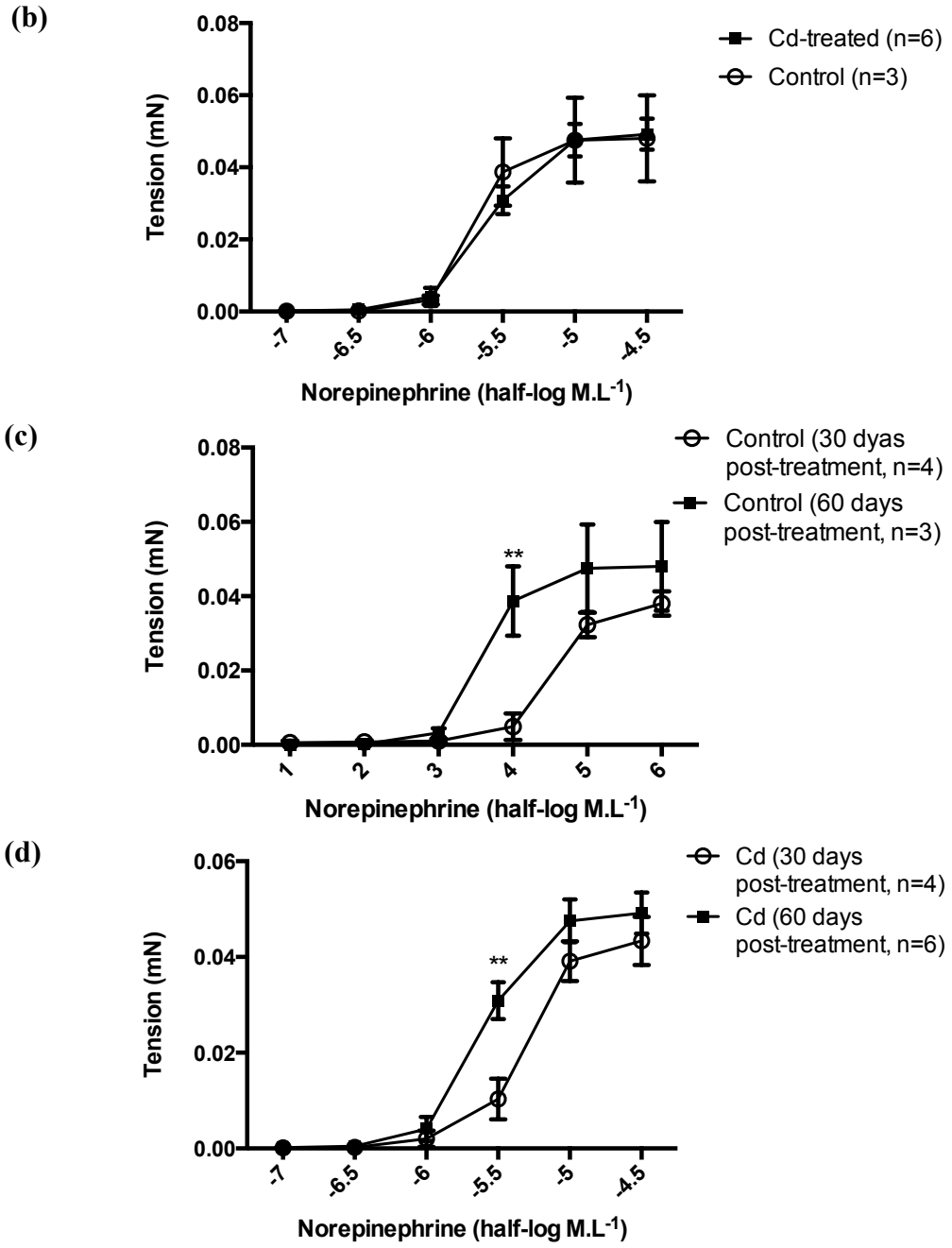


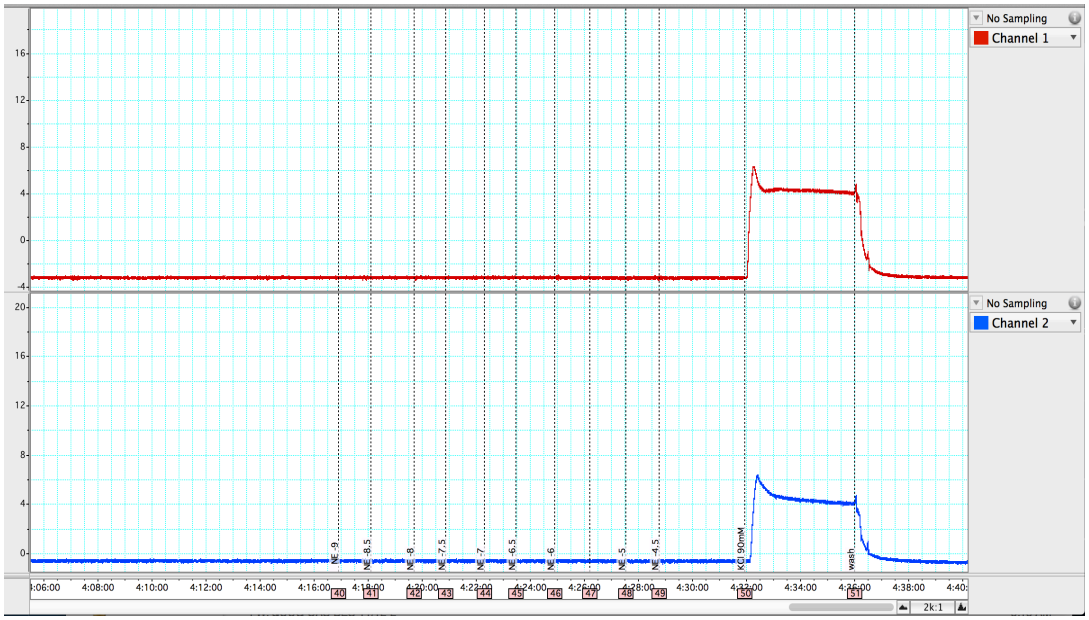
Figure 16: The effect of Cd exposure on concentration response curve to Norepinephrine (NE): control vs Cd-treated 30 days post-treatment (a), control vs Cd-treated 60 days post-treatment (b), control 30 days post-treatment vs control 60 days post-treatment (c), Cd-treated 30 days post-treatment vs Cd-treated 60 days post-treatment (d) Values are means ± SEM. $^{**}P<0.01$.

Table 12: The 50% effective concentrations (EC_{50}) for control and Cd-treated in response to norepinephrine (NE), 30 days and 60 days post-treatment.

	Control	**Cd-treated**
30 days post-treatment	-5.224 ± 0.096	-5.358 ± 0.119
60 days post-treatment	-5.673 ± 0.023	-5.589 ± 0.041

To determine the effect of Cd on other alphas receptors such as α_2 AR, mesenteric arteries were pre-treated with Prazosin (selective α_1 AR antagonist). The response was diminished in both groups in response to NE after 30 and 60 days of treatment (fig.17).

(a)



(b)

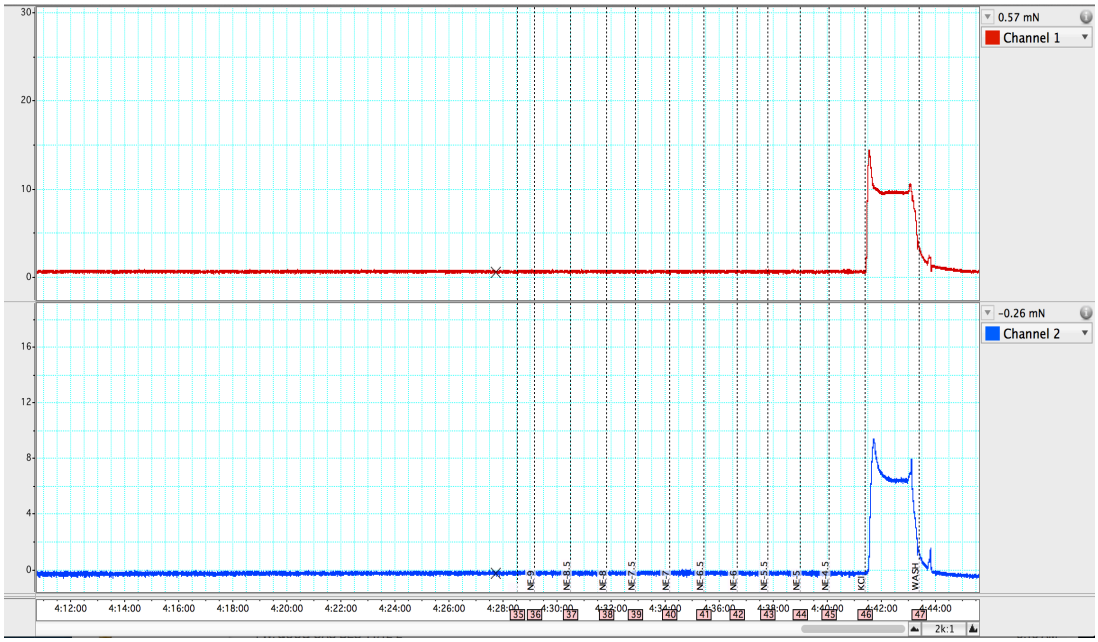


Figure 17: Vascular response to norepinephrine (NE) in presence of α_1 AR antagonist (prazosin, 30min before NE). (a) 30 days post-treatment. (b) 60 days post-treatment.

CHAPTER 4: DISCUSSION

Cardiovascular diseases (CVDs) are one of the main causes of death worldwide. Several risk factors contribute to the etiology of CVDs such as tobacco smoke, hyperlipidemia, atherosclerosis, hypertension and environmental pollution. Cadmium (Cd) is one of the most harmful environmental pollutants and has been associated with high blood pressure. Although many pathways have been tested to explain Cd contribution to hypertension, little is known about the involvement of α_1 AR and phenotypic modulation of VSMCs. In our study we investigated the possible role of the α_1 AR receptors and phenotypic modulation of VSMCs as a key players in hypertension.

Alpha ARs are highly distributed among the vascular tree of the human body. α_1 AR is mostly expressed in the VSMCs compared to other cell types. Due to its presence in the VSMCs, α_1 AR is the major regulator of the vascular tone. High expression of α_1 AR is positively correlated with the pathogenesis of CVDs. As we mentioned above, Cd is a dangerous pollutant revealed to be a contributor in the elevation of blood pressure.

In this report, our results showed that Cd significantly increased the expression of α_1 AR in concentration and time dependent manner. This suggests that α_1 AR could be one of the mechanisms that mediate Cd-induced hypertension. Physiologically, α_1 AR is activated through EP or NE binding to induce contraction. EP and NE are locally regulated via an enzyme called Catechol-*O*-methyltransferase enzyme (COMT). This enzyme has been showed to be inhibited due to Cd toxicity leading to an increase in the serum and urine level of EP and NE (101). Cd also appears to augment the vascular responses to NE (106). Taken together, this means that by inactivating COMT and thus

increasing catecholamine levels in the blood, Cd may be increasing the pressor responses to EP. As we show here, Cd also increased the expression of α_1 AR. This could be one underlying mechanism by which Cd potentiated the VSMC contraction. However, further experiments are warranted before such a conclusion can be made.

Gene expression can be controlled via transcription regulation, or post-transcriptional regulation. Here, we show that the α_1 AR -induced expression is due to a transcriptional activation, evident by the absence of induction when cells were pretreated with actinomycin D, a DNA-dependent RNA synthesis inhibitor. Transcriptional regulation of α_1 AR could be modulated by several parameters such as: hypoxia, ischemic reperfusion, growth factors, cyclic adenosine monophosphate (cAMP) and catecholamine stimulation (27). Catecholamines, of which NE is one, induces the m-RNA level of α_1 AR under chronic stimulation in the neonatal rat cardiomyocytes (108). The regulation of transcriptional action is initiated through intracellular signal transduction such the cAMP-PKA pathway. Interestingly, we found that the observed significant increase of α_1 AR expression was abolished in the presence of H89, a PKA inhibitor. This suggests that transcriptional activity of α_1 AR was mediated via the PKA pathway. Once PKA is activated by cAMP, PKA is translocated to the nucleus where it activates cAMP response element-binding protein (CREB). CREB is a transcription factor that binds to DNA and can act to switch on the gene expression of α_1 AR. Evidence indicates that activation of α_1 AR could increase cAMP level in some cells (109). Other evidence indicates that α_1 AR stimulation leads to the phosphorylation of CREB (27). Taken together, it is possible that Cd induce the α_1 AR expression through the stimulation of α_1 AR, which increases the

cAMP level (109), inside the VSMC leading to the activation of PKA as we showed in our results. Finally PKA activation stimulates the CREB phosphorylation (27), to switch on the α_1 AR transcription and m-RNA production. This positive feedback regulation in response to Cd toxicity could explain how relatively very small amount of Cd precipitates various diseases, particularly hypertension. However, further experimentation is warranted in order to validate this apparently vicious signaling loop.

The excited findings from the *in vitro* work were applied to further investigation by *in vivo* studies. Wistar rat was our normotensive model in this study because it is the most commonly used rat model in CVDs research. Food intake, body weight and water intake were monitored in order to determine the effect of Cd on the normal metabolic parameters. Our results revealed that Cd has no direct effect on food intake and body weight of the rats. However, a high significant decrease in water intake was observed in the Cd-treated rats compared to control, which could be due to the water taste. This significant reduction in water consumption of the Cd-treated group did not influence the body weight gain; this could be due to the observed increase in the amount of visceral fat. Functional studies were applied to investigate the vascular α_1 AR sensitivity under Cd treatment, using phenylephrine and NE, selective and non-selective α_1 AR agonists. We found that vascular reactivity was slightly affected. Cd shifted the phenylephrine concentration-response curve to the left (not statistically significant) in the mesenteric arteries isolated from Cd-treated rats after 30 days of treatment. However, Cd didn't show any enhancement after 60 days treatment. Further investigations were conducted in order to examine the effect of NE on Cd-induced vascular α_1 AR activity. The results

were similar to phenylephrine response after 30 and 60 days. Conflicting findings were reported recently by Almenara et al. (2013), who reported a greater increase of the contraction response to phenylephrine in the aortic rings of Cd-treated rat ($100mg.L^{-1}$) for 4 weeks (50), which represent 30 days in our study. The conflict of results could be explained by the response effect occurs after 30 days but not 60 days may be due to the long period of Cd exposure in relevance to the accumulative dose that may suppresses the sensitivity of the α_1 AR. This explanation can be supported by finding reported by Skoczyńska (1997) about the potentiation vasoconstriction effect of NE on isolated rat mesenteric artery treated with 2.85 mg/kg/day (106). The type of tested vessels could contribute to the difference in the response between our result and the result of Almenara et al. Ordinarily, due to the size differences between aorta and mesenteric arteries, aorta exhibits more force than mesenteric artery and even the expression of α_1 AR may be higher in the aorta. Additionally, we were also compared between the control groups and treated groups to see the time-dependent differences between them. A shift to the left was observed in both control and treated groups after 60 days of treatment comparing to 30, in response to phenylephrine. While in response to NE higher augmentation was resulted and showed to be significant at dose $10^{-5.5}$ $M.L^{-1}$. These findings could be an age-related factor due to the increase in animal age. Increase in blood pressure is highly expected in aged people. It has been reported that aging is associated with the elevation in plasma catecholamines level (110), as well as α_1 AR expression (111). Beside the α_1 AR role in VSMCs contraction, we were interested to look at role of other type of alpha ARs such as α_2 AR. This was examined by blocking the α_1 AR with its antagonist prazosin. The

response curve to NE was diminished after blocking the α_1 AR. This means α_2 AR has no role in mediating VSMCs contraction under Cd toxicity.

The present study did not show any significant change in values of SBP, DBP, HR or MAP between control and Cd-treated. The vascular reactivity data support these results, whereas other conflicting data reported that Cd significantly increased the SBP, DBP and the MAP of mice treated with 100 mg/l $CdCl_2$ via drinking water for 8 weeks (24). This could be related to the species of the animal and Cd dose. Here we used rat instead of mice, and higher Cd dose compared to 100mg/l.

This apparent contradiction in our data between the *in vitro* (α_1 AR expression) and *in vivo* (α_1 AR activity) could be related to various factors. Species differences between the two models may be one of them. In the *in vitro* we used the human primary cell line (HASMCs) where rats were used as a model for the *in vivo*. Thus, Cd may induce the expression of α_1 AR in human cells but not in rat tissues. Moreover, *in vivo* where more physiological exist other parameter may influence or modulate the effect rather than the *in vitro* setting. In the *in vitro* Cd target the VSMCs directly, where in the *in vivo* Cd crosses different barriers to reach the VSMCs.

Phenotypic modulation of VSMCs is a normal physiological function in response to vessel damage. Under certain pathologic conditions, phenotypic modulation could have an important role in narrowing the blood vessel, which would then drastically impact blood pressure. Our study aimed also to study the effect of Cd on the VSMCs phenotypic modulation as a factor of vascular pathogenesis underlying hypertension. Hypertrophy (increase in cell area), and hyperplasia (increase in cell volume) are critical

in narrowing blood vessel. Our results illustrated that Cd prompt the hypertrophy of HASMCs as it was shown in the significant increase of cell area at 1000nM Cd treatment. Along with these findings, Mikhaleva et al. (1991) found that chronic Cd exposure in rats causes hypertrophic effects in the arterial wall as well as the left ventricular cardiomycetes (86). From the viability test, Cd didn't show any signs of cell proliferation, which result in hyperplasia. In contrast, Cd has been reported to increase cell proliferation at 100nM or less in bovine and rabbit aortic smooth muscle cells (92). This could be related to species differences.

VSMCs migration was studied, which is a well-known phenomenon of atherosclerosis. In the present study, results revealed that Cd induces HASMCs migration, which is a main contributor in the formation of the neointima. Normally, the formation of neointima started by ECs injury or dysfunction. As a result, injured ECs express adhesion molecules such as: ICAM-1 and VCAM-1 to facilitate the migration of monocyte to reach the intima. Once these monocytes arrive to the intima, they start to secret inflammatory cytokines and growth factors to stimulate VSMCs migration from the medial layer to the intima, where they would proliferate forming neointima. Under Cd toxicity, the number of leukocytes increased significantly in Cd-treated mice (88). In addition, Cd showed to induce the expression of adhesion molecules (ICAM-1) and (VCAM-1) (87) and the secretion of inflammatory cytokines (89), (90). Additionally, the disruption of the extracellular matrix is one of the key players in VSMCs migration. Matrix metalloproteinases (MMPs) are enzymes that have a role in this action. They catalyze the connective tissues and extracellular matrix to induce migration. Indeed,

MMPs expression showed to be elevated in Cd-treated rat (93). Moreover, the cellular signal transduction that mediates VSMCs migration needs to be verified. An evidence showed that extracellular signal-regulated kinases (ERK1/2) were involved in VSMCs migration after pre-treatment with AngII (112). Equally important, $CdCl_2$ reported to increase ERK1/2 in a biphasic manner in SHR VSMCs. Furthermore, Cd showed to induce the phosphorylation of ERK1/2 in pancreatic b-Cells (113). This suggests that Cd stimulates VSMCs migration via ERK1/2 signaling pathway.

Vascular senescence is another type of vascular remodeling that occurs normally in aged people. Due to its health impact on vascular pathogenesis and hypertension, our findings showed that Cd promotes HASMCs senescence in a concentration dependent manner. Senescent VSMCs are commonly found in atherosclerotic plaque (33). The presence of senescent cells in atherosclerotic plaque may affect the stability of the plaque and leads to a serious complication of atherosclerosis, which is the plaque rupture. Indeed, senescence can also induced via cellular stress such as oxidative stress (33). Vascular senescence can be recognized by a number of markers. These markers include: increase in ROS production, positive to β-galactosidase activity and expression of cell cycle arrest proteins. Supportive evidence showed that Cd induces oxidative stress and the generation of free radicals and reactive oxygen species (59), (60). It has been revealed that Cd increased the generation of intracellular ROS in pancreatic b-Cells and the phosphorylation of p38 protein as a sign of cell cycle arrest (113). This suggests that Cd induces ROS production, which may then activate signal transduction resulting in p38 phosphorylation leading to inhibition of cell growth. More investigations are needed,

however, to clarify the mechanism of Cd-induced VSMCs senescence.

Generally, phenotypic modulation can be triggered due to various factors and stimuli. During this process, VSMCs phenotype shifted from contractile phenotype to a synthetic phenotype. This shift is characterized by changes in gene expression. In this regard, these changes include silencing of contractile gene expression and up-regulation of other functional genes that are responsible to stimulate synthetic phenotype such as: migration, cell proliferation, hypertrophy and senescence. Therefore, gene expression can be suggested as a future target of therapeutic approach in Cd-induced vascular phenotypic modulation. One of the suggested directions is using the micro-RNAs (miRNAs), which is a critical regulator of gene expression (114). Another factor can be involved in the initiation of VSMCs phenotypic modulation, which is the destruction of cell surface heparan sulfate. Heparan sulfate is a glycoprotein present on the cell membrane and has a role in the organization of actin filaments and stabilization of cell morphology. Thus, degradation of cell surface heparan sulfate may induce VSMCs phenotype by affecting actin organization and activation of gene expression (115). Hence, Cd-induced phenotypic modulation is critical and plays a role in vascular pathogenesis including hypertension (our focus in this study). It needs more attention and complete understanding of the underlying mechanisms that initiate it. To our knowledge, our findings regarding the effects of Cd on HASMCs phenotypic modulation have never been addressed before, so we are the first in reporting descriptive findings on HASMCs phenotypic modulation. The underlying mechanisms that mediate HASMCs phenotypic modulation are our future direction in order to have a clear and solid image.

The interpretation of these results direct us to consider some limitations. The number of mesenteric arteries used in the vascular reactivity experiment could be one of the most important limitations. As shown in the results, both response curves (phenylephrine and norepinephrine) were shifted to the left, but the difference was not statistically different. Therefore, number of animals could be a factor at the 30 days tested time, where if the number increased this could enhance the response curve more than what we obtained. This could be reflected from our capabilities in having a team that can help in the *in vivo* part, which needs extra time and efforts. Beside the limitations, the strength of our study can be shown from the combination between two fields, which are the *in vitro* and *in vi*vo to help in presenting the complete picture. Excitingly, we are the first who report the direct effect of Cd on the expression of α_1 AR and the signaling pathway that mediate this expression, as well as the phenotypic modulation of HASMCs. To our knowledge, no one has reported these findings before.

CHAPTER 5: CONCLUSION

In conclusion, our results dissect a novel pathway employed by cadmium to increase expression of vascular α_1 AR a major player in hypertension, via transcriptional-dependent mechanism. Moreover, this expression of α_1 AR was diminished when cells were pre-incubated with H89, a protein kinase A (PKA) inhibitor. This indicates that PKA plays an important role in mediating the Cd-induced expression of α_1 AR. Functional studies did not show any significant effect on vascular reactivity in response to α_1 AR agonists as well as blood pressure. In addition Cd showed to be highly involved in VSMCs phenotypic modulation. It has induced hypertrophy, migration and senescence. These modulations associated with many CVDs including hypertension. This new paradigm offers a better understanding and thus potential improvement of pollution-related CVDs. Subsequently, the findings of this study can be applied in Qatar due to high prevalence of CVDs among Qatari population, thus Cd could be one of the contribution risk factors in this health problem. Because Qatar is facing a rapid urbanization and increase in industrial activities especially in the last few years, more studies should be conducted to determine the level and the sources of Cd contamination.

Future Directions

This thesis formulated a comprehensive data on a proposed mechanism of Cd-induced hypertension. Our work opens up some paths for future work in Cd toxicity and vascular diseases including hypertension. We suggest that Cd induce the expression of α_1 AR as a blood pressure modulator, however the expression of each subtype of α_1 AR needs to be investigated. Here we agreed that Cd up-regulate the α_1 AR expression, but

the functional activity of the receptor was not tested *in vitro*. This can be easily tested by treating the HASMCs with 1000nM $CdCl_2$ for 48 hrs and then examine the cells response to EP and NE. The reactivity can be recorded by video camera under inverted microscope. Results can be analyzed by using ImageJ software. α_2 ARs are located pre-synaptic at the nerve ends and have a role in the regulation of EP and NE secretion. The direct effect of Cd on the expression of α_2 ARs is also an interesting point to elucidate.

In the *in vivo* studies, we showed that the mesenteric artery intoxicated with 15mg/kg/day did not significantly enhance the vascular activity, so we are interested in looking at the effect of Cd on the aorta responsiveness. It has been reported that Cd lead to endothelial damage and dysfunction (77), therefore vasodilation activity may have a role in modulating the vascular tone. More supportive studies on the vasodilation of the vascular bed under Cd toxicity can be addressed by looking at the expression of eNOS and bioavailability of NO. Histological studies also can help visualizing possible damage of the vascular wall that may result from Cd toxicity.

In the present study, we reported the descriptive results of VSMCs phenotypic modulation. We showed that Cd induced VSMCs migration, hypertrophy and senescence. Whereas, the underlying mechanisms that mediates phenotypic modulation needs to be clarified. Different signaling pathways may be involved. It has been reported that the 3 Mitogen Activated Protein (MAP) kinases such as; c-Jun NH2-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 kinases had a role in VSMCs phenotypic modulation like migration, proliferation and gene expression (116). These MAP kinases are highly important to be validated to figure out the mechanisms of Cd-

induced VSMCs phenotypic modulation. Investigating the underlying mechanisms of Cd-induced phenotypic modulation will be significant for future therapeutic inventions.

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