# THE EFFECTS OF NANOSILVER ON VASCULAR REACTIVITY AND ENDOTHELIAL FUNCTION ON ISOLATED RABBIT CAROTID ARTERY

BY

EHANIRE OSAYUWARE VANESSA

MAT NO. PG/MED1001247

DEPARTMENT OF PHYSIOLOGY,

SCHOOL OF BASIC MEDICAL SCIENCES,

COLLEGE OF MEDICAL SCIENCES,

UNIVERSITY OF BENIN,

BENIN CITY.

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ABSTRACT

Nanoparticles (Nano particles) are defined as structures with a diameter less than 100 nm and novel physical and chemical properties that differ sharply from the macro forms. The medical use of nanosilver particles is growing mainly due to their antimicrobial properties. The impact of NSPs on the regulation of vascular tone (vasoconstriction/vasodilation), blood flow distribution, heartbeat, electric mechanisms, etc.; as well as their protective or adverse role in the development and progression of cardiovascular pathologies has not been properly understood. The aim of this present work was to determine the effect of nanosilver on vascular reactivity in isolated rabbit carotid artery. The study involved standard laboratory Organ Bath procedures. Twenty milli/liter organ baths containing PSS, bubbled with 95% $O_2$, 5% $CO_2$ and maintained at 37$^{o}$C and pH 7.4. The recording system is a 4-channel Grass Polygraph. Cumulative dose response tests to agonists phenylephrine (PE), was examined separately, in normal PSS (control). Contractile responses were analysed with reference to maximal contractions induced by 80 mM $K^+$ in normal PSS. In another experiment, arterial rings were precontracted with $EC_{70}$ M PE and /or high potassium PSS (80 mM $K^+$). At stable contraction, cumulative relaxation response to nanosilver was studied. The above protocol was repeated in endothelium intact (+E) (control) and endothelium denuded (-E) rings. In another experiment, arterial rings were precontracted in 20 mM $K^+$. At a stable contraction, cumulative relaxation response to nanosilver was studied. Cumulative dose response tests to phenylephrine was examined in normal PSS (control) and following 20 minutes exposure to varying concentrations of nanosilver solution (1.25 mcg/ml and 2.50 mcg/ml) in rabbit carotid rings (+E or –E). Contractile responses were analysed with reference to maximal contractions induced by 80 mM $K^+$. Dose relaxation tests to acetylcholine was examined in normal PSS

(control), following 5 minutes exposure to L name or indomethacin and 20 minutes exposure to nanosilver solution (1.25 mcg/ml) in rabbit carotid rings. Relaxation responses were analysed with reference to maximal contraction induced by EC70 M PE in normal PSS. Data was presented as Mean ± SEM. Graphs and statistical analysis were done using Graphpad prism version 7.03 and Student t-test. P-value ≤ 0.05 were considered statistically significant. The results show that nanosilver induced attenuated contractile response in varying concentrations in +E and –E carotid rings. The results also show that nanosilver induced relaxation is endothelium dependent, and nanosilver showed a biphasic effect. In conclusion, the study shows that NSPs has a biphasic effect on vascular tone and NSPs induced relaxation is endothelium dependent. Further studies should be carried out to determine the mechanism of action of NSPs and the adverse effects associated with NSPs.

CHAPTER ONE

# INTRODUCTION

Nanoscience and nanotechnology have received much significance since the last few years and metal nanoparticles are at the leading edge of swiftly developing a field of nanotechnology. The theory of nanotechnology was found in 1959 by the physicist Richard P. Feynman who received the Nobel Prize in this field. Nanoparticles can be defined as a "particulate dispersal or solid particles with a size in the range of 10-1000 nm" (Czajka, 2005). One nanometer spans 3-5 atoms lined upon in a row (Czajka, 2005). Nanoparticles are of enormous scientific interest as they are efficiently a bridge between bulk materials and atomic or molecular structures (Czajka, 2005).

Regardless of their conventional benefits, current studies have shown the toxicological effects of nanosilver particles under certain conditions (Xia *et al.,* 2008). To be precise, the toxic effects in different cell types depend on the concentration and distribution patterns of the nanoparticles (Xia *et al.,* 2008). The number of nanoparticles has a great influence in the binding and activation of membrane receptors and subsequent protein expression in cancer cells (Asharani *et al.,* 2012). Rosas-Hernández *et al.,* worked on the dose-dependent effects of chemically prepared nanosilver particles with an average size of 45 nm in coronary endothelial cells, particularly studying biological effects such as cell proliferation and nitric oxide (NO) production. They concluded that nanosilver particles had a dual effect with regards to cell proliferation, whereby proliferation was inhibited at low concentrations of nano particles and stimulated at high concentrations (Rosas-Hernández *et al*., 2009). Trickler *et al.,* (2010) investigated blood–brain barrier

inflammation and permeability in primary rat brain micro vessel endothelial cells using variable sizes of nanosilver (25, 40, or 80 nm nanosilver particles). This study concluded that smaller sizes induced significant effects at all concentrations and time points. The substance in nano-regime demonstrates an enormous number of new properties. The properties of substances in nano size are entirely different from the equivalent atomic and molecular counterparts. The improvement in the field of nanoscience has brought about ground-breaking changes in industrial, medicinal, textile, food packaging and agriculture fields (Muller and Bohm, 1998; Esenaliev, 2000; Thote and Gupta, 2005; Wagner *et al.,* 2006; Emerich and Thanos, 2007).

A study from rat brain endothelial cell culture suggests that AgNP toxicity depends on particle size, surface area, dose, and exposure time (Grosse *et al.,* 2013). New biological characteristics have been deliberated to these materials in multiple laboratories, establishing that nanosilver particles are capable of producing angiogenic/antiangiogenic, vasodilation/vasoconstriction, pro-oxidant/antioxidant, cytotoxic, apoptotic and phagocytic effects, and some of these effects depend on the concentration, size, biological target and exposure time (Schrand *et al.,* 2010; Trickler *et al.,* 2010; Kang *et al.,* 2011; Haase *et al.,* 2012; Grosse *et al.,* 2013).

Considering the various applications of NSPs and their possible effects in the body, this study was designed to evaluate the effects of nanosilver on the vascular reactivity and endothelial function in isolated rabbit carotid artery.

## 1.2 AIM

The aim of the present study was to determine the effect of nanosilver on vascular reactivity and endothelial function on isolated rabbit carotid artery.

## 1.3 SPECIFIC OBJECTIVES

- To study the effects of variations of nanosilver on dose response to phenylephrine on the contractile responses

- To examine the effects of nanosilver in ach-induced relaxation and endothelial function and possible mechanism of vascular action.

## 1.4 RESEARCH QUESTIONS

- Does nano silver alter significantly the contractile responses to phenylephrine in vascular smooth muscles of rabbit carotid artery?

- Is nanosilver mode of action receptor or non-receptor dependent?

- Is the vascular relaxant effect of nanosilver endothelium dependent?

# LITERATURE REVIEW

## NANOSILVER

Silver nanoparticles (nanosilver particles or nano silver) material has been receiving increasing attention in the recent decade. It has been reported that nanosilver has a history of approximately 120 years of usage and was known as 'colloidal silver' (Nowack *et al.,* 2011). Nanotechnology is basically science, engineering and technology conducted at the nano scale which is about 1 to 100 nm (Mihail, 2011). The U.S. National Nanotechnology Initiative (NNI) described it as understanding and managing of matter at dimensions between approximately 1 and 100 nanometers where unique phenomenon enables novel application (Mihail, 2011).

Several studies which have been conducted in the past years showed that these materials act entirely different from their bulky counterparts in terms of the optical, electronic and catalytic properties, etc. (Niemeyer 2001; Sun and Xia 2002; Moore 2006). Its properties are believed to be strongly associated with the shape, size and substructure of metal nanoparticles. It was then stated that these characteristics of nanoparticles could be finely regulated by controlling these factors (Sun and Xia 2002; Xia *et al.,* 2008).

Lok *et al.,* (2013) in his study reported that nanosilver particles displayed deterioration of the outer membrane and split of the plasma membrane, thereby causing depletion of intracellular ATP. Silver which has a higher affinity to react with sulphur or phosphorus-containing biomolecules in the cell, would preferably bind to sulphur containing proteins in the membrane or inside the cells and

elements containing phosphorus (like DNA). Numerous studies have reported that Nano silver can alter the permeability of the cell membrane to K+ and also to Na+ at a concentration that does not limit Na+ K+ ATP activity or mitochondrial function (Kone *et al.,* 1988).

**Antibacterial properties**

The antibacterial effect of NSPs is broad on a range of Gram-negative and Gram-positive bacteria and antibiotic resistant bacteria strains (Kim *et al*., 2007). The antimicrobial efficacy of NSPs solely depends on their size and concentration. Normally, a high concentration of nano silver antimicrobial activities are more effective, while small sizes of the particle can kill bacteria at a lower concentration. Aside from size and concentration, shape can also influence the antimicrobial efficiency of NSPs. In an investigation by Sadeghi *et al*., (2012), the antimicrobial activity of different nanosilver shapes, which include silver nano-plates, silver nanorods, and silver nanoparticles, on Staphylococcus aureus and E. coli were studied and they found the antimicrobial activity of nano plates were the best. It has been reported that the combination of NSPs with various antibiotics have better antimicrobial effects compared with NSPs or antibiotics alone. Li *et al*., (2005), in an experiment found a greater antibacterial effect on E. coli when amoxicillin and silver nanoparticles were combined compared to when they were applied separately.

Though nanosilver and its antimicrobial effect have been widely studied, the exact mechanism of action of NSPs is still subtle. It has been accepted that NSPs can bind to and subsequently enter the bacterial cell wall, and thereby cause structural changes of the cell membrane and thereby increase cell permeability, leading to cell death (Sondi and Salopek-Sondi, 2004). Another potential mechanism is the development of free radicals and subsequent free radical-induced membrane

damage, which was examined by Kim *et al*., (2007). It has been revealed that NSPs can release silver ions and bind with the thiol groups of many vital enzymes and phosphorus-containing bases, thereby inhibiting some functions in cells, such as the prevention of cell division and DNA replication (Matsumura *et al*., 2003). In addition, NSPs might control signal transduction by altering the phosphotyrosine profile of bacterial peptides for the potential antibacterial mechanism

**Antifungal properties**

Nanosilver act as an effective antifungal agent against a broad spectrum of common fungi. An investigation by Kim *et al*., (2008), NSP antifungal properties on a total of 44 strains of six fungal species were investigated and found that NSPs can prevent the growth of Candida albicans, Candida glabrata, Candida parapsilosis, Candida krusei, and Trichophyton mentagrophytes effectively. It was observed that NSPs can disrupt cellular membrane and prevent the normal budding process; but, the exact mechanisms of action of nanosilver against fungi are still not understood (Nasrollahi *et al*., 2011; Kim *et al*., 2009).

**Antiviral properties**

NSPs are also known for its antiviral activities against HIV-1 (Sun *et al*., 2005), hepatitis B virus (Lu *et al*., 2008), respiratory syncytial virus (Taylor *et al*., 2005), herpes simplex virus type 1 (Baram-Pinto *et al*., 2009), and monkeypox virus (Rogers *et al*., 2008). Studies have shown that NSPs have higher antiviral activity than silver ions, which is due to species difference as they dissolve to release Ag0 (atomic) and Ag+ (ionic) clusters, however silver salts release Ag+ only (Taylor *et*

*al*., 2005). Studies show that the anti-HIV mechanism of nanosilver is based on the inhibition of the initial stages of the HIV-1 cycle (Lara *et al*., 2010). It was shown that NSPs can bind to glycoprotein (gp)120, and then inhibit clusters of differentiation (CD) 4-dependent binding, fusion, and infectivity. They also act as an effective virucidal agent that blocks HIV-1 cell-free and cell-associated infection. However, NSPs shown inhibitory effect to post-entry stages of the HIV-1 life cycle (Lara *et al.,* 2010).

**Anti-inflammatory properties**

NSPs also show anti-inflammatory properties. For instance, in a model involving swine with contact dermatitis induced by topically applying 1,2-dinitrochlorobenzene, nanosilvers improved the expression of proinflammatory cytokines transforming growth factor-β and tumor necrosis factor-α (Nadworny *et al*., 2008). In an experiment, Shin and Ye found that NSPs reduced nasal symptoms in allergic rhinitis mice and repressed OVA-specific immunoglobulin E, IL-4, and interleukin-10, and that inflammatory cell infiltration and goblet cell hyperplasia were inhibited by nanosilvers (Shin and Ye, 2012). In a human clinical study, wound dressing containing NSPs promoted the healing of chronic leg ulcers by not only reducing bacteria numbers in the wound bed, but by decreasing inflammatory response as well. NSPs' ability to reduce cytokine release and matrix metalloproteinases (Castillo *et al*., 2008) decrease lymphocyte and mast cell infiltration, and induce apoptosis in inflammatory cells, (Shin and Ye, 2012) may explain their anti-inflammatory mechanisms.

ROUTE OF EXPOSURE; NANOSILVER

The main routes of exposure of NP are inhalation, dermal contact and ingestion; and also, systemic administration. (Lankveld *et al*., 2010; Chen and Schluesener, 2008). Irrespective of the route of exposure, their shared characteristics is that the circulatory system is accountable for distributing the Nano particles that have trans located to the blood stream to the rest of the body, and it is also important to take note of the preferential accumulation and distribution sites and structures of nanosilver particles following accumulation in the body. Exposure routes gain additional relevance to the cardiovascular system (CVS) since potential AgNP biomedical applications involved in the prevention, prognostic and treatments of diseases are still under evaluation, and one requirement is obligatory maintenance in the circulatory system, in order for the nanosilver particles to reach targeted cells and tissues (Moghimi *et al*., 2001). Irrespective of exposure route, the concerns of the toxic effects (side, local and systemic toxic) of nanosilver particles is growing. nanosilver particles are now been incorporated into hygiene sprays and health products (Luoma, 2008), in respect to their advantage to act as an external stimulus which is able of activate the protection mechanisms, which also activates inflammatory processes in order to produce proinflammatory cytokines and promote the release of cytotoxic molecules (Ricciardolo *et al*., 1997; Sung *et al*., 2008, 2009; Español *et al*., 2010; Gonzalez *et al*., 2011; Ahamed *et al*., 2010). The products containing nanosilver are applied directly to the nasal or oral cavity, and thus, the Nano particles can be directed to the lungs, establishing immediate

contact with the blood flow (Chen and Schluesener, 2008). Using studies of inhalation of nanosilver particles as a case study for bio-distribution, the study demonstrated that the lungs were an initial target, as well as the brain, Nano particles reaches the brain through the nasopharyngeal system, previous deposit in the olfactory mucosa (Oberdörster *et al*., 2005). For instance, inhaled silver ultrafine particles are phagocytosed by alveolar macrophages and aggregates can be generated, persisting for approximately 7 days. It was also reported that the aggregates of nanosilver particles (25 nm–1 μm) can be cytotoxic to both alveolar macrophages and epithelial cells on the lungs, which suggest respiratory complications. Since Nano particles has the ability to pass through the air–blood barrier, recent studies indicate that, once inhaled and deposited, nanosilver particles can translocate to interstitial sites and distribute to several organs such as brain, liver and kidney (Geiser *et al*., 2005; Oberdörster *et al*., 2004; Sung *et al.*, 2008). Furthermore, a predictive computational model suggests that inhaled Nano particles may accumulate along the respiratory tract (Asgharian and Price, 2007) depending on the size and local geometry within the respiratory system. For instance, large (bigger than 1 μm) and very small (smaller than 2 nm) particles tend to deposit in the nasopharyngeal compartment, whereas intermediate-sized Nano particles (2–100 nm) deposit in the alveolar and tracheobronchial compartments (Quadros and Marr, 2010; Oberdörster *et al*., 2005).

In a research performed by Takenaka *et al*., (2001) nanosilver particles (15 nm) were inhaled or instilled in rats, the lung particle content decreased rapidly in respect to time, and the nanosilver particles were detected in blood and other organs, such as, liver, kidney and brain. Interestingly, the study demonstrated a higher NP accumulation in the female kidney versus that of the male kidney; however, the mechanisms behind the differences remain unclear (Takenaka *et al*.,

2001). In addition, a 90-day prolonged inhalation exposure of nanosilver particles (18 nm) in rats showed sub-chronic inhalation toxicity, which affected the liver and lungs (Ji *et al*., 2007; Sung *et al*., 2009), and caused a decrease in tidal and minute lung volumes, and also inflammatory responses such as mixed inflammatory cell infiltration, chronic alveolar inflammation, bile duct hyperplasia around the central vein to the hepatic lobule with infiltration of inflammatory cells, including eosinophils (Sung *et al*., 2008).

Reports, together with pharmacokinetic studies of nanosilver particles, have indicated that pulmonary, dermal and oral absorption of these nano-materials is low and depends on the size, coating, dose and route of exposure (Lin *et al*. 2014). In order to increase the bioavailability of nanosilver particles, proper coating and smaller size is desirable. However, regardless of the route of exposure, nanosilver particles are mainly located in the spleen and liver depending on the particle size, since smaller Nano particles tend to locate in the liver, whereas larger particles are located in the spleen, as the liver has fenestrate pores smaller than 100 nm and therefore, can internalize smaller particles. On the other hand, the spleen absorbs larger particles with higher affinity (Lin *et al*., 2014).

**Effects of Nanosilver On the Cardiovascular System**

Presently, there are insufficient reports showing the effect of nanosilver particles in physiological models that investigate and suggest possible mechanisms of action when Nano particles enter into circulation and interact with different kinds of blood vessels directly. However, the impact of nanosilver on the regulation of vascular tone (vasoconstriction/vasodilation), blood flow distribution, heartbeat, etc.; as well as their protective or adverse role in the development and progression

of cardiovascular pathologies has not been fully understood (Rosas-Hernandez *et al*., 2009).

Presently, numerous studies have focused on the effects of nanosilver particles on different types of cells in the vascular system however, the results have been contradictory (Cui and Gao, 2003; Rosas-Hernandez *et al*., 2009), studies performed have provided evidence that suggest that Nano silver particles have dual and opposite effects on blood structure, angiogenesis and permeability and these effects depend on the size, surface chemistry, concentration of the Nano particles, target organ. In addition, the inadequate studies that have been performed in complex models of blood vessels and due to the fact that majority of them are concentrated on the angiogenic process prevents the creation of a definite conclusion about the concern that nanosilver particles are beneficial or harmful for human health. Moreover, the mechanisms of action of the biological interactions of nanosilver particles in the vascular system are still not well understood (Cui and Gao 2003; Rosas- Hernandez *et al*., 2009).

Biological characteristics have been convened to the materials in several laboratories, demonstrating that nanosilver particles are able of produce angiogenic/antiangiogenic, vasodilation/vasoconstriction, pro-oxidant/antioxidant, cytotoxic, apoptotic and phagocytic effects, and majority of these effects are dependent on the concentration, size, biological target and exposure time (Cui and Gao 2003; Rosas- Hernandez *et al*., 2009). Still, the side effects of Nano particles upon the vascular system and the bio-distribution need to be investigated under normal conditions and also pathological conditions which could change the transport of Nano particles, through the vessel wall, organelle-targeted delivery, and other effect on the vascular cells (Kabanov 2006; Lankveld *et al*., 2010; Ahamed *et al*., 2010).

In an in vitro study, which involved micro vascular-derived coronary endothelial cells (CEC), nanosilver particles induced an opposite effect which was dependent on the concentration. At concentrations less than 10 μg/ml, nanosilver particles (45 nm) induced cytotoxic effects, at a concentration of 50 μg/ml and higher, nanosilver particles produced a NO-dependent cellular proliferation. Recent investigations have revealed that the proliferation was due to the activation of the endothelial nitric oxide synthase (eNOS), it was phosphorylated at serine residue 1,177, (represents the active form of the enzyme) (Rosas-Hernandez *et al*., 2009). The endothelial NO-induced proliferation occurred at a high concentration of nanosilver particles, in the same pattern to other endogenous vasoactive substances, such as ACh, BK or vascular endothelial growth factor (VEGF); (Venema *et al*., 1996; Gonzalez *et al*., 2004).

The PVP-coated nanosilver particles produced angiogenesis in an in vitro study through the formation of the endothelial tube; and both angiogenic steps' effects were facilitated by the production of NO and vascular endothelial growth factor (VEGF). However, since nanosilver particles targeted VEGF, further studies into the growth factor pathway was vital. The VEGF pathway was stimulated, through increasing the phosphorylation of Akt and extracellular signal regulated kinase (ERK1/2).

It is well know that mitogenic and angiogenic effects induced by VEGF are facilitated in part by the NO production, as a result of eNOS activation, that activated by the phosphorylation of Akt, a mechanism that generates NO, which in turn is linked with the proliferation signaling induced by VEGF, since NO stimulates 3′-5′ cyclic guanosine monophosphate (cGMP) production and activation of cGMP-dependent protein kinase (PKG), leading finally to MAPK activation (Clapp *et al*., 2006; Garcia *et al*., 2008). This mechanism suggests the

role of nanosilver particles as exogenous vasoactive stimulus for NO production with mitogenic properties.

## PHYSIOLOGY OF THE VASCULAR SMOOTH MUSCLE

Vascular smooth muscle cells (VSMCs) represent dynamic systems constantly changing their shape and architecture through the coordinated rearrangement of cellular components and position within the vascular wall to adapt to alterations in their chemical and mechanical environments (Martinez-Lemus *et al*. 2009). The major types of blood vessels are: arteries, arterioles, capillaries and veins. There are no smooth muscle cells in the capillaries but the muscular component of the others is an important aspect of their normal function. Vessels differ by their physical dimensions, morphology, function and muscular composition. The one feature all blood vessels have in common is that they are all lined with endothelial cells. This includes the capillaries (which are essentially made of endothelial cells) and the pulmonary vessels (Clark and Pyne-Geithman, 2005).

Arteries are the blood vessels that carry blood away from the heart and generally are exposed to greater pressures than other vessels. For this reason, they are thicker than other vessels with a larger compliment of extracellular proteins; collagen and elastin. Many arteries can stretch up to half their length via these passive elements. Stretching of the arteries by the heart's pumping action allows the vessels to 'store' the heart's energy, which is returned to the vascular system between heartbeats. If

the stored energy (called passive elastic recoil) from the heart were not stored in the arteries, the blood flow would stop between each beat, resulting in discontinuous blood flow. With the passive elastic recoil, however, the blood flow and pressure does not fall to zero and the tissues can be supplied with a constant supply of blood. Passive elastic recoil of the arteries has several important benefits to the
cardiovascular system;
(1) it helps to promote laminar flow,
(2) it does not require much metabolic energy from the vessels and
(3) it produces a continuous flow of blood throughout the vascular tree (Clark and Pyne-Geithman, 2005).

The continuous blood flow is important because arteries are damaged by turbulent flow and they are also responsible for producing laminar flow in the vessels. They, therefore, are somewhat responsible for their own pathological changes regarding vascular damage due to turbulent flow. Lastly, because passive elastic recoil does not require energy in the form of contractile activity, the vessels do not utilize ATP to produce this 'contraction' of the vessels. It therefore keeps the efficiency of blood delivery high and means that there is a contractile reserve should it be required.
Knowing that the arteries have such passive elastic recoil means that one cannot consider the blood vessels as a 'rigid pipe'. Arteries have an important role in the function of the cardiovascular system, which is dependent upon its contractile, metabolic and passive properties. To understand vascular smooth muscle function and dysfunction therefore, one needs to understand each of these integral parts of the artery (Clark and Pyne-Geithman, 2005).

The vascular system is sometimes referred to as a vascular tree because it branches from its 'root' at the aorta to many smaller parts. Coming from the heart as the aorta and branching into arteries, arterioles, capillaries then venules and finally veins, it goes from un-branched, to highly branched and back to the two branches of the vena-cava. The branching from the aorta to the capillaries produces a large increase in surface area for the flow of blood, which decreases the velocity of flow. Decreasing the blood flow in the capillaries facilitates oxygen and metabolite delivery. Vascular smooth muscle functions to achieve and maintain adequate and controlled flow of blood to the tissues (Clark and Pyne-Geithman, 2005).

Vascular smooth muscle has a relatively unique ability to maintain tension with relatively low ATP consumption using a mechanism called Latch (Dillon *et al*., 1981). The latch mechanism is where the vessel's cross bridges remain attached under tension but without being phosphorylated. This results in a slowly cycling cross bridge that consumes about seven-fold less ATP to maintain tension at a constant length. Therefore, vascular smooth muscle is very economical when it maintains tension because of a low cost for maintaining latch bridges (Clark and Pyne-Geithman, 2005).

The Carotid Artery

The carotid artery is located adjacent to vital neurovascular structures and is responsible for supplying adequate blood flow to the brain. The cervical segment (C1) of the internal carotid artery begins at the bifurcation of the common carotid artery. The cervical carotid is located adjacent to cranial nerves IX, X, XI, and XII and the sympathetic chain. After the bifurcation of the common carotid artery at

the level of hyoid, the C1 internal carotid artery travels deep to the mandible to enter the skull base medial to the styloid process through the carotid canal. As opposed to the external carotid artery (ECA) with its multiple branches, the ICA is devoid of arteries, which can be a useful feature during an open approach for identification with confidence (Thomas *et al*., 2014).

## VASCULAR SMOOTH MUSCLE CONTRACTION

### $Ca^{2+}$-dependent myosin light chain phosphorylation

Smooth muscle myosin differs from skeletal and cardiac myosins in that it lacks intrinsic myosin ATPase activity in the pure state. Smooth muscle myosin requires a posttranslational modification, phosphorylation of Ser 19 of the 20-kDa regulatory light chain to display enzymatic activity. This phosphorylation is caused by a dedicated Ser/Thr kinase, myosin light chain kinase (MLCK). (Ito and Hartshorne, 1990).

Myosin Light Chain Kinase (MLCK) is a Ca/CaM-dependent kinase and is most simply activated by increases in cytoplasmic ionized Ca ($[Ca^{2+}_i]$) levels such as occurs with a large number of G-protein coupled receptor-mediated agonists, such as alpha agonists or by depolarization of the cell membrane by channel activity or experimentally by equimolar replacement of NaCl with KCl in physiologic saline

solution. It has also been reported that increases in the free CaM level (Hulvershorn *et al*., 2001) or Ca-independent changes in the kinase activity of MLCK can also occur by phosphorylation-mediated events (Kim *et al*., 2000).

The crossbridge cycle describes the development of force through a series of complexes between actin (A), myosin (M), ATP, and its hydrolysis products, ADP and Pi (Sweeney and Houdusse, 2010). Beginning in the rigor state (AM), ATP binding to AM results in rapid dissociation of AM, forming an A+M-ATP state, and then ATP is hydrolysed by myosin. After hydrolysis, the crossbridge enters a weakly attached, pre-power stroke AM-ADP-Pi state, and then transitions to a strongly bound, force producing AM-ADP-Pi state. After Pi release from the AM-ADP-Pi state, the crossbridge enters a AM-ADP state, which then isomerizes to a high force generating state (AM-ADP) followed by ADP release and returning to the rigor state (AM). MgATP subsequently binds to the AM state, causing rapid crossbridge detachment, and then another crossbridge cycle commences. The duty cycle is defined as the proportion of time crossbridges spend in strongly attached states divided by the time for the total crossbridge cycle (De La Cruz and Ostap, 2004); high duty cycle motors are capable of processive movement (i.e., dynein, myosin V), whereas skeletal muscle myosin has a low duty cycle that prevents the development of an internal load from strongly bound crossbridges, which would decrease shortening velocity. Although the crossbridge cycle for all types of myosin is frequently described in this generic manner, differences exist between the kinetics of skeletal, cardiac, and smooth muscle and even within different smooth muscle tissues, requiring changes in the crossbridge cycle to explain the differences in AMATPase rates (Rosenfeld *et al*., 2000).

## REGULATION OF VASCULAR SMOOTH MUSCLE FUNCTION

Vascular smooth muscle cells (VSMC), like all other muscle cells, depend on $Ca^{2+}$ influx to initiate contraction. However, the VSMC intracellular $Ca^{2+}$ concentration does not only determine the contractile state, but also affects the activity of several $Ca^{2+}$ dependent transcription factors and thereby determines VSMC phenotype. To govern the various $Ca^{2+}$-dependent functions and in reaction to different stimuli, VSMCs use a variety of plasmalemmal and sarcoplasmic reticulum (SR) $Ca^{2+}$ channels to produce a large repertoire of $Ca^{2+}$ signals, which differ in their spatial and temporal distribution (Amberg and Navedo, 2013). These signals range from cell-wide changes in $[Ca^{2+}]$ to highly localized $Ca^{2+}$ entry or release events. $Ca^{2+}$ can enter the cell from the extracellular space or be released from the largest intracellular $Ca^{2+}$ store, the sarcoplasmic reticulum (SR). Extracellular $Ca^{2+}$ influx is mainly mediated by the opening of voltage dependent L-type $Ca^{2+}$ channels (LTCC), but there are a number of other channels that modulate intracellular $Ca^{2+}$, including transient receptor potential (TRP) cation channels. Because of their high single-channel conductance and expression in VSMCs, LTCCs have the largest influence on global $[Ca^{2+}]_i$, and their activity largely determines the VSMC's contractile state and ultimately vessel diameter (Knot and Nelson, 1998).

### Vascular Endothelium and Function

The endothelium was once thought of as the "cellophane wrapper" of the vascular tree, with no other specific functions than affording selective permeability to water and electrolytes (Wilson and Lerman, 2001). However, enormous advances since the 1980's have led to an understanding of the complex functions of this large

endocrine organ. Vascular endothelial cells line the entire circulatory system, from the heart to the smallest capillaries. These cells have very distinct and unique functions that are paramount to vascular biology. These functions include fluid filtration, such as in the glomeruli of the kidneys, blood vessel tone, hemostasis, neutrophil recruitment, and hormone trafficking. Researchers in vascular biology know well that the endothelium embodies a wide range of homeostatic functions (Durand and Gutterman, 2013), with the ability to act in both sensory and effector capacities. The role of the endothelium is affected through the presence of membrane-bound receptors for numerous molecules including proteins, lipid-transporting particles, metabolites, and hormones, as well as through specific junctional proteins and receptors that govern cell-cell and cell-matrix interactions (Douglas *et al*., 1998). Endothelial cells (EC's) also play a pivotal role in regulating blood flow. In part, this role is achieved due to the capacity of quiescent EC's to generate an active antithrombotic surface that facilitates the transit of the plasma and cellular constituents throughout the vasculature. Perturbations, such as those that may occur at sites of inflammation or high hydrodynamic shear stress, disrupt these activities and induce EC's to create a prothrombotic and antifibrinolytic microenvironment. Blood flow is also regulated, in part, through secretion and uptake of vasoactive substances by the endothelium that act in a paracrine manner to constrict and dilate specific vascular beds in response to stimuli such as endotoxin (Patel, 2001). The endothelium is a cell layer lining the blood luminal surface of vessels. It was until recently considered to be just a lining, but it is now realized that EC's have important functions besides merely providing a lining for vessel walls.

The vascular endothelium serves as the interface between the blood and the enclosing vascular structures. This unicellular layer serves a number of crucial

roles in physiology and pathophysiology, including regulating vascular tone and promoting a local anti-inflammatory and anti-fibrotic milieu. The endothelium also contributes to the regulation of systemic metabolism by virtue of actions of fuel substrates, insulin, and other regulatory factors directly at the level of the vascular endothelium (Kieren, 2013).

## ENDOTHELIAL FUNCTIONS

### Thrombosis and thrombolysis

The endothelium plays a crucial role in providing the proper haemostatic balance. The function of endothelial cells far exceeds that of providing a non-thrombogenic inner layer of the vascular wall that helps to maintain blood fluidity. Under physiological conditions, endothelial cells prevent thrombosis by means of different anticoagulant and antiplatelet mechanisms. These cells are involved in all main haemostatic pathways triggered upon vascular injury and limit clot formation to the areas where haemostasis is needed to restore vascular integrity (Stern, 1991).

### Coagulant mechanisms

Endothelial cells form the luminal vascular surface and thus have a central role in the regulation of coagulation. One important way in which endothelial cells control the clotting system is by regulating the expression of binding sites for anticoagulant and procoagulant factors on the cell surface. In the quiescent state, endothelial cells maintain blood fluidity by promoting the activity of numerous anticoagulant pathways, including the protein C/protein S pathway. After activation, as can be brought about by cytokines, the balance of endothelial properties can be tipped in favor of clot formation through the coordinated induction of procoagulant and suppression of anticoagulant mechanisms. Tumor necrosis factor suppresses the formation of throbomodulin, an endothelial anticoagulant cofactor, and induces the expression of tissue factor, which is a procoagulant cofactor (Atherton and Born, 1972).

**Regulation of vascular tone and growth**

Endothelial cells play an important regulatory role in the circulation as a physical barrier and as a source of a variety of regulatory substances. Endothelium-derived nitric oxide and prostacyclin are released in response to physical stimuli, hormones, and platelet-derived substances and induce vascular relaxation and inhibit platelet function. Certain substances can evoke a hyperpolarization of smooth muscle cells. In addition, endothelial cells can release several contraction-inducing factors (e.g., endothelin, thromboxane A2, angiotensin II, superoxide, and unidentified endothelium-derived contraction-inducing factors), at least under certain conditions. Endothelial cells are also a source of growth inhibitors and promoters, such as heparin and heparin sulphates, platelet-derived growth factor, and

thrombospondin. Several vasoactive substances produced by the endothelium, such as nitric oxide, endothelin, and angiotensin II may also play a role in the regulation of vascular growth. Thus, the endothelial layer can regulate vascular tone and growth. Dysfunction of these endothelium-dependent regulatory systems may play a role in cardiovascular diseases, such as hypertension and atherosclerosis (Barton *et al*., 2012).

**Cell Proliferation and Angiogenesis**

The endothelium also involved in blood vessel formation. The development of a functional vascular network requires a remarkable degree of coordination between different cell types undergoing complex changes and is exquisitely dependent upon signals exchanged between these cell types. Vascular endothelial growth factor (VEGF) provided the first example of a growth factor specific for the vascular endothelium. More recently, an entirely unrelated family of growth factors known as the angiopoietins (Ang) and particular members of the very large ephrin family have been identified as having unique effects on the endothelium. Recent insights have led to a model of vascular formation that attempts to incorporate the known vascular-specific growth factors. According to this model, VEGF is the most critical driver of vascular formation, as it is required to initiate the formation of immature vessels by vasculogenesis or angiogenic sprouting. Ang1 and ephrinB2 are subsequently required for further remodeling and maturation of this initially immature vasculature, notably as endothelial cells integrate with supporting cells such as smooth muscle cells and pericytes. Following vessel maturation, Ang1 seems to continue to be important for maintaining the quiescence and stability of the mature vasculature (Michiels, 2003).

Vasoactive Agents

Vasoactive agents are a group of bioactive chemicals, which change vasomotor tone through their influence on various peripheral receptors. Most of these agents have inotropic effects (e.g. norepinephrine) as they bind with receptors positioned on the surface of the myocardium. Some pharmacologic agents are difficult to classify as their effects overlap, however all vasoactive drugs affect stroke volume and heart rate. This determines cardiac output and, as a consequence, overall cardiovascular function (Ino *et al*., 2006). In the ideal situation, inotropic drugs would only have effects directly on myocardial cells, without simultaneous stimulation of other receptors. As one quickly learns, such is not the case with many of our most important vasoactive agents. Some inotropic agents such as dopamine (depending on the dose) provoke vasoconstriction while other such agents, like isoprenaline, cause vasodilatation. Pure vasoconstrictors produce arterial wall constriction by stimulation of α-adrenergic receptors (e.g. phenylephrine) or by stimulating V1a receptors (e.g. vasopressin) on the vascular endothelial surfaces. Pure vasodilators, on the other hand, are classified according to their predominate effect on the arteries (e.g. hydralazine), veins (e.g. nitroglycerine), or both arterial and venous (e.g. nitropruside) vessels (Ino *et al*., 2006).

Vasoactive agents comprise broad categories of drugs that have vasoactive effects. These include but not limited to inotropes, vasopressors, vasodilators and inodilators (Zhongheng and Kun, 2016). Dopamine and norepinephrine are the most commonly used vasoconstrictor in the initial phase of septic shock. Dopamine in a large dose activates α1 receptor and has potent vasoconstriction effects. Norepinephrine has great potency in increasing blood pressure via α1

receptor, but it's in constriction effect is not so potent as dopamine. Epinephrine has equivalent effect on heart and vasculature (Zhongheng and Kun, 2016).

Phenylephrine is a potent vasoconstrictor without significant effect on cardiac function. The highly selective action site of phenylephrine makes it unique in all vasopressors. From hemodynamic perspective, it increases mean arterial pressure, but lowers cardiac output. There is no effect on heart rate. As compared with norepinephrine, phenylephrine has no additional beneficial effect on cardiopulmonary performance and global oxygen transport (Zhongheng and Kun, 2016).

**Pharmacological properties of vasoactive medications**

| **Vasoactive agents** | **Heart** | **Vasculature** | | **Others** | **Hemodynamic effect** |
|---|---|---|---|---|---|
| | **β1** | **α1** | **α2** | | |
| Dopamine (dose >5 μg/kg/min) | 0~3+ | 0~3+ | 0~2+ | – | CO↑; MAP↑; HR↑↑ |
| Dobutamine | 4+ | + | 2+ | – | CO↑↑; MAP↔; HR↑ |
| Adrenalin | 4+ | 2~4+ | 1~3+ | – | CO↑↑; MAP↑↑; HR↑↑ |
| Norepinephrine | 2+ | 4+ | 1+ | – | CO↔; MAP↑↑; |

| | | | | | |
|---|---|---|---|---|---|
| Phenylephrine | 0 | 4+ | 0 | – | HR↑<br>CO↓; MAP↑; HR↔ |
| Vasopressin/terlipressin | 0 | 0 | 0 | Vasoconstriction via vasopressin receptor | CO↓; MAP↑; HR↔ |
| Milrinone | 0 | 0 | 0 | Phosphodiesterase-3 inhibition | CO↑↑; MAP↓↔; HR↑ |

(Zhongheng and Kun, 2016)

**Autonomic Receptors**

The efferent pathways of the ANS consist of 2 neurons that transmit impulses from the CNS to the effector tissue. The preganglionic neuron originates in the CNS with its cell body in the lateral horn of the gray matter of the spinal cord or in the brainstem. The axon of this neuron travels to an autonomic ganglion located outside the CNS, where it synapses with a postganglionic neuron. This neuron innervates the effector tissue (Laurie, 2007).

Synapses between the autonomic postganglionic neuron and effector tissue—the neuroeffector junction—differ greatly from neuron-to-neuron synapses. The postganglionic fibers in the ANS do not terminate in a single swelling like the synaptic knob, nor do they synapse directly with the cells of a tissue. Instead, where the axons of these fibers enter a given tissue, they contain multiple swellings called varicosities. When the neuron is stimulated, these varicosities release neurotransmitters along a significant length of the axon and, therefore, over a large surface area of the effector tissue (Laurie, 2007)

## Divisions of the Autonomic Nervous System

The ANS is composed of 2 anatomically and functionally distinct divisions, the sympathetic system and the parasympathetic system. Both systems are tonically active. In other words, they provide some degree of nervous input to a given tissue at all times. Therefore, the frequency of discharge of neurons in both systems can either increase or decrease. As a result, tissue activity may be either enhanced or inhibited. This characteristic of the ANS improves its ability to more precisely regulate a tissue's function (Laurie, 2007).

## Sympathetic Division

The preganglionic neurons of the sympathetic system arise from the thoracic and lumbar regions of the spinal cord (segments $T_1$ through $L_2$). Most of these preganglionic axons are short and synapse with postganglionic neurons within ganglia found in the sympathetic ganglion chains. These ganglion chains, which run parallel immediately along either side of the spinal cord, each consist of 22 ganglia. The preganglionic neuron may exit the spinal cord and synapse with a postganglionic neuron in a ganglion at the same spinal cord level from which it arises. The preganglionic neuron may also travel more rostrally or caudally (upward or downward) in the ganglion chain to synapse with postganglionic neurons in ganglia at other levels. In fact, a single preganglionic neuron may synapse with several postganglionic neurons in many different ganglia. the preganglionic neuron may travel to the adrenal medulla and synapse directly with this glandular tissue. The cells of the adrenal medulla have the same embryonic origin as neural tissue and, in fact, function as modified postganglionic neurons.

Instead of the release of neurotransmitter directly at the synapse with an effector tissue, the secretory products of the adrenal medulla are picked up by the blood and travel throughout the body to all of the effector tissues of the sympathetic system (Laurie, 2007).

**Parasympathetic Division**

The preganglionic neurons of the parasympathetic system arise from several nuclei of the brainstem and from the sacral region of the spinal cord (segments S2-S4). The axons of the preganglionic neurons are quite long compared to those of the sympathetic system and synapse with postganglionic neurons within terminal ganglia which are close to or embedded within the effector tissues. The axons of the postganglionic neurons, which are very short, then provide input to the cells of that effector tissue (Laurie, 2007).

The preganglionic neurons that arise from the brainstem exit the CNS through the cranial nerves. The occulomotor nerve (III) innervates the eyes; the facial nerve (VII) innervates the lacrimal gland, the salivary glands and the mucus membranes of the nasal cavity; the glossopharyngeal nerve (IX) innervates the parotid (salivary) gland; and the vagus nerve (X) innervates the viscera of the thorax and the abdomen (eg, heart, lungs, stomach, pancreas, small intestine, upper half of the large intestine, and liver). The physiological significance of this nerve in terms of the influence of the parasympathetic system is clearly illustrated by its widespread

distribution and the fact that 75% of all parasympathetic fibers are in the vagus nerve. The preganglionic neurons that arise from the sacral region of the spinal cord exit the CNS and join together to form the pelvic nerves. These nerves innervate the viscera of the pelvic cavity (eg, lower half of the large intestine and organs of the renal and reproductive systems) (Laurie, 2007).

## Neurotransmitters of the Autonomic Nervous System

The 2 most common neurotransmitters released by neurons of the ANS are acetylcholine and norepinephrine. Neurotransmitters are synthesized in the axon varicosities and stored in vesicles for subsequent release. Nerve fibers that release acetylcholine are referred to as cholinergic fibers. These include all preganglionic fibers of the ANS, both sympathetic and parasympathetic systems; all postganglionic fibers of the parasympathetic system; and sympathetic postganglionic fibers innervating sweat glands. Nerve fibers that release norepinephrine are referred to as adrenergic fibers. Most sympathetic postganglionic fibers release norepinephrine (Laurie, 2007).

## Receptors of The Autonomic Nervous System

The neurotransmitters of the ANS and the circulating catecholamines bind to specific receptors on the cell membranes of the effector tissue. All adrenergic receptors and muscarinic receptors are coupled to G proteins which are also embedded within the plasma membrane. Receptor stimulation causes activation of

the G protein and the formation of an intracellular chemical, the second messenger. (The neurotransmitter molecule, which cannot enter the cell itself, is the first messenger.) The function of the intracellular second messenger molecules is to elicit tissue-specific biochemical events within the cell which alter the cell's activity. In this way, a given neurotransmitter may stimulate the same type of receptor on 2 different types of tissue and cause 2 different responses due to the presence of different biochemical pathways within each tissue (Laurie, 2007).

| Tissue | Sympathetic Receptor | Sympathetic Stimulation | Parasympathetic Stimulation |
|---|---|---|---|
| Eye | | | |
| Radial muscle of iris | $\alpha_1$ | Contraction (dilation of pupil; mydriasis) | – |
| Sphincter muscle of iris | | – | Contraction (constriction of pupil; miosis) |
| Ciliary muscle | $\beta_2$ | Relaxation for far vision | Contraction for near vision |
| Heart | $\beta_1$, $\beta_2$ | ↑ Heart rate | ↓ Heart rate |
| | | ↑ Force of contraction | ↓ Rate of conduction |
| | | ↑ Rate of conduction | |
| Arterioles | | | |
| Skin | $\alpha_1$ | Strong constriction | – |
| Abdominal viscera | $\alpha_1$ | Strong constriction | – |
| Kidney | $\alpha_1$ | Strong constriction | – |
| Skeletal muscle | $\alpha_1$, $\beta_2$ | Weak constriction | – |
| Spleen | $\alpha_1$ | Contraction | |
| Lungs | | | |
| Airways | $\beta_2$ | Bronchodilation | Bronchoconstriction |
| Glands | $\alpha_1$, $\beta_2$ | ↓ Secretion | ↑ Secretion |
| Liver | $\alpha_1$, $\beta_2$ | Glycogenolysis | – |
| | | Gluconeogenesis | – |
| Adipose tissue | $\beta_3$ | Lipolysis | – |
| Sweat glands | Muscarinic; | Generalized sweating | – |
| | $\alpha_1$ | Localized sweating | – |
| Piloerector muscles | $\alpha_1$ | Contraction (erection of hair, goose bumps) | – |
| Adrenal medullae | Nicotinic | ↑ Secretion of epinephrine, norepinephrine | – |
| Salivary glands | $\alpha_1$, $\beta_2$ | Small volume $K^+$ and water secretion | Large volume $K^+$ and water secretion; amylase secretion |
| Stomach | | | |
| Motility | $\alpha_1$, $\beta_2$ | Decreased | Increased |
| Sphincters | $\alpha_1$ | Contraction | Relaxation |
| Secretion | | | Stimulation |
| Intestine | | | |
| Motility | $\alpha_1$, $\beta_2$ | Decreased | Increased |
| Sphincters | $\alpha_1$ | Contraction | Relaxation |
| Secretion | | | Stimulation |
| Gallbladder | $\beta_2$ | Relaxation | Contraction |
| Pancreas | | | |
| Exocrine | $\alpha$ | ↓ Enzyme secretion | ↑ Enzyme secretion |
| Endocrine (Islets β cells) | $\alpha$ | ↓ Insulin secretion | ↑ Insulin secretion |
| Urinary bladder | | | |
| Detrusor muscle (bladder wall) | $\beta_2$ | Relaxation | Contraction |
| Urethra sphincter | | Contraction | Relaxation |
| Kidney | $\beta_1$ | ↑ Renin secretion | – |

(https://www.ncbi.nlm.nih.gov/corecgi/tileshop/tileshop.fcgi?p=PMC3&id=414011&s=8&r=3&c=2)

## MATERIALS AND METHODS

### 2.2. PHYSIOLOGICAL SALT SOLUTION (PSS)

The constituent salts listed below were obtained from the Department of Physiology, University of Benin, Benin city, Edo State and were used throughout the experimental procedures to constitute normal PSS. Stock solutions of the salts except $NaHCO_3$ and glucose were made in distilled water. The solution was thoroughly mixed and stored in the refrigerator. Working PSS was prepared just prior to use from the refrigerated stock solutions and kept at $37^0$C.

| | | |
|---|---|---|
| I. | NaCl: | 58.44 g in 1L of distilled water. |
| II. | KCl: | 74.55 g in 1L of distilled water |
| III. | $KH_2PO_4$ | 136.07 g in 1L of distilled water |
| IV. | $MgSO_4$ | 246.91 g in 1L of distilled water |
| V. | $CaCl_2$ | 147.7 g in 1L of distilled water |

## 2.2.1. COMPOSITION OF PSS

| 1 litre Salts | Normal PSS | Low $HCO_3$ PSS |
|---|---|---|
| NaCl | 119.0 ml | 150.0 ml |
| KCl | 4.7 ml | 5.4 ml |
| $KH_2PO_4$ | 1.2 ml | 1.2 ml |
| $MgSO_4$ | 1.2 ml | 1.2 ml |
| $CaCl_2$ | 1.6 ml | 5.0 ml |
| $NaHCO_3$ | 1.25 g | 0.3 g |
| $C_6H_{12}O_6$ | 2 g | 2 g |

**Table 1: Different constituents and compositions of PSS used for the experiment.**

Normal PSS was used basically to test for the viability of the endothelium using $10^{-5}$M ACH to induce relaxation responses.

## 2.3. DRUGS

$EC_{70}$ ($10^{-7}$M) phenylephrine or 20 mM or 80 mM high potassium was used to induce maximum contraction. Relaxation responses tested were to $10^{-5}$M Ach and nanosilver to rings treated with indomethacin or L name

## 2.4. EXPERIMENTAL ANIMAL

Mature male and female rabbits were used throughout the experiment. These were purchased from a Rabbitry in the University of Benin, Benin-city, Edo State. They were used the day they were purchased.

### 2.4.1. TISSUE ISOLATION

The carotid arteries were carefully dissected, cleaned free of adhering connecting tissues and cut into ring segments of about 2 mm in length. The prepared rings were suspended between two S-shaped steel holders hung vertically in the 20 ml organ bath containing PSS, with the lower holder attached to the notch at the bottom of the organ bath and the upper holder to the hooks on the transducers. The PSS was continuously bubbled with 95% $O_2$ and 5% $CO_2$ gas mixture and was maintained thermostatically at $37^0$C.

## RECORDING SYSTEM

Standard organ bath apparatus was made use of. The carotid artery ring preparation was connected to Grass Model Ft. 03 transducer for recording of isometric contraction and relaxation on a 4-channel Grass model polygraph, at a resting tension of 2 g. The point of application of each agent was clearly indicated on the recording sheet. All other treatments given to the tissue (e.g washes) and relevant

experimental information (e.g. chart speed) were clearly indicated on the recording sheet.

GRASS MODEL POLYGRAPH

ORGAN BATH APPARATUS

**EXPERIMENTAL PROTOCOLS**

a) ESTABLISHING THE VIABILITY OF THE ENDOTHELIUM

One of the rings of the carotid artery has its endothelium destroyed by passing through and rubbing the rough edges of a focep on the lumen of the strips. Another ring strip cut has its endothelium intact. Viability of the endothelium was established in normal PSS.

b) DOSE RESPONSE TO PHENYLEPHRINE (PE)

After mounting the ring preparations in the organ bath containing normal PSS, they were allowed to equilibrate for a 60 minutes period. $10^{-7}$ M phenylephrine was added to the bath aimed at inducing contraction.

c) DOSE RESPONSE TO $10^{-5}$M ACETYLCHOLINE (ACH) CONCENTRATION AFTER PE PRE- CONTRATION

Ring preparations with intact endothelium were precontracted with $10^{-7}$ M PE. When the contractions were stable, ACH of $10^{-5}$ M concentration was added to the organ bath, to induce relaxation.

The results of the protocol above were estimated as a percentage of the initial PE pre-contraction.

% Relaxation = magnitude of relaxation x 100

Magnitude of contraction

d) TO DETERMINE THE POSSIBLE MECHANISM OF ACTION

Arterial rings were precontracted with $EC_{70}$ M PE and /or high potassium PSS (80 mM $K^+$). At stable contraction, cumulative relaxation response to nanosilver was studied. The protocol was repeated in endothelium intact (+E) (control) and endothelium denuded (-E) rings.

e) TO EXAMINE THE CONTRIBUTION OF EDHF OR POTASSIUM CHANNEL IN THE EFFECT OF NANO SILVER

In another experiment to induce the possible mechanism of action of nano silver, arterial rings were precontracted in 20 mM $K^+$. At a stable contraction, cumulative relaxation response to nanosilver was studied.

f) TO CHARACTERISE NANO SILVER MECHANISM OF RELAXATION

Dose relaxation tests to acetylcholine was examined in normal PSS (control), following 20 minutes exposure to nano silver (1.25 mcg/ml) only, 5 minutes exposure to L name ($1x\ 10^{-5}$ M) or indomethacin ($3 \times 10^{-6}$ M) and 20 minutes exposure to nanosilver solution (1.25 mcg/ml) in rabbit carotid rings. Relaxation responses were analysed with reference to maximal contraction induced by EC70 M PE in normal PSS.

g) TO EXAMINE NANO SILVER CONTRACTILE EFFECTS

Cumulative dose response tests to phenylephrine was examined in normal PSS (control) and following 20 minutes exposure to varying concentrations

of nanosilver solution (1.25 mcg/ml and 2.50 mcg/ml) in rabbit carotid rings (+E or –E). Contractile responses were analysed with reference to maximal contractions induced by 80 mM $K^+$.

## STATISTICAL ANALYSIS

All results are presented as Mean ± SEM. Graphs and statistical analysis were done using Graphpad prism version 7.03 and Student t-test. P-values ≤ 0.05 were considered statistically significant. $EC_{50}$ And $IC_{50}$ (concentrations producing 50% maximal response) values were determined graphically.

# Chapter four

# Results

The results are presented as mean ± standard error of the mean.

This figure represents the dose response to phenylephrine.

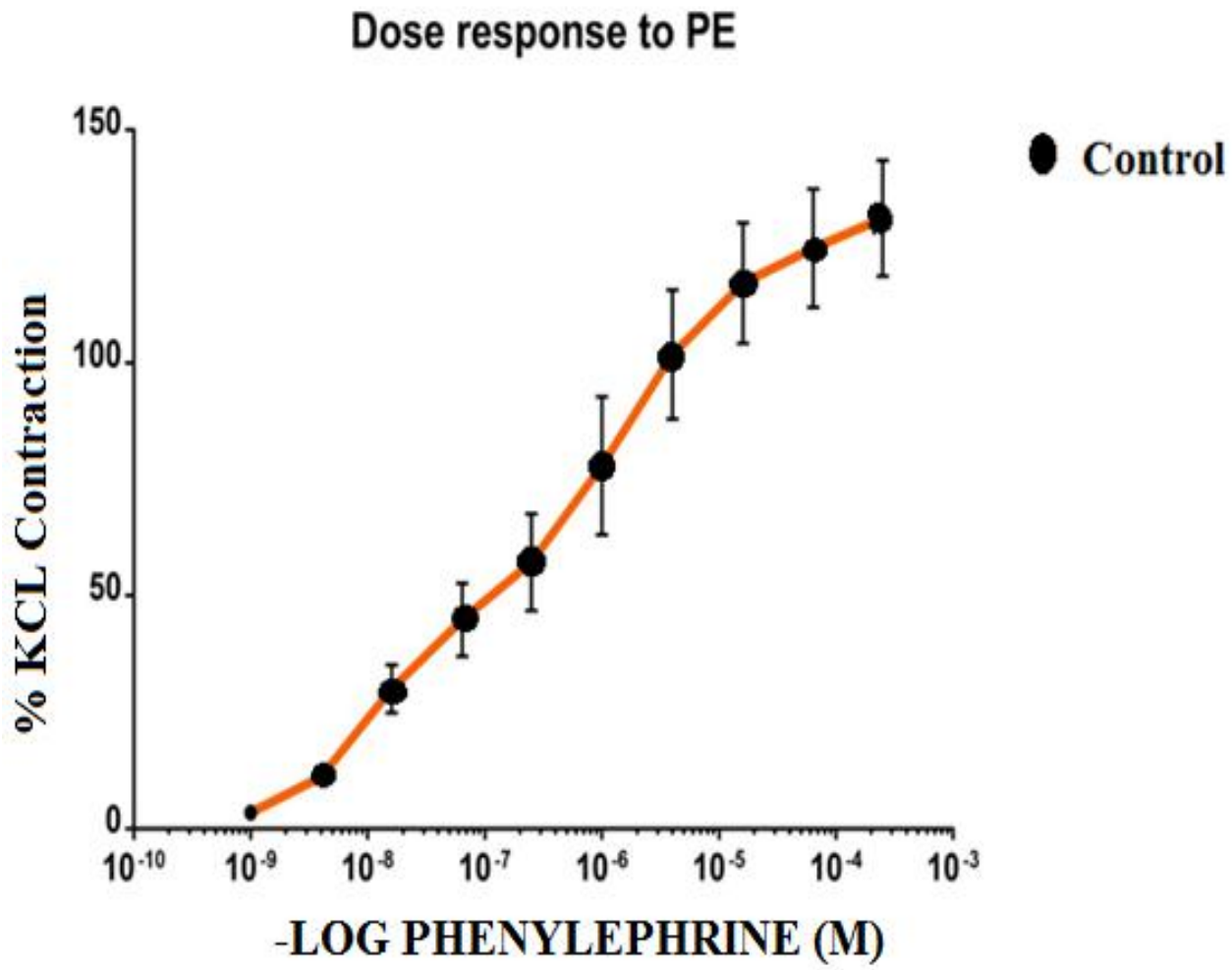


**Figure 1a**: shows a dose response curve.

Dose Relaxation to Ach

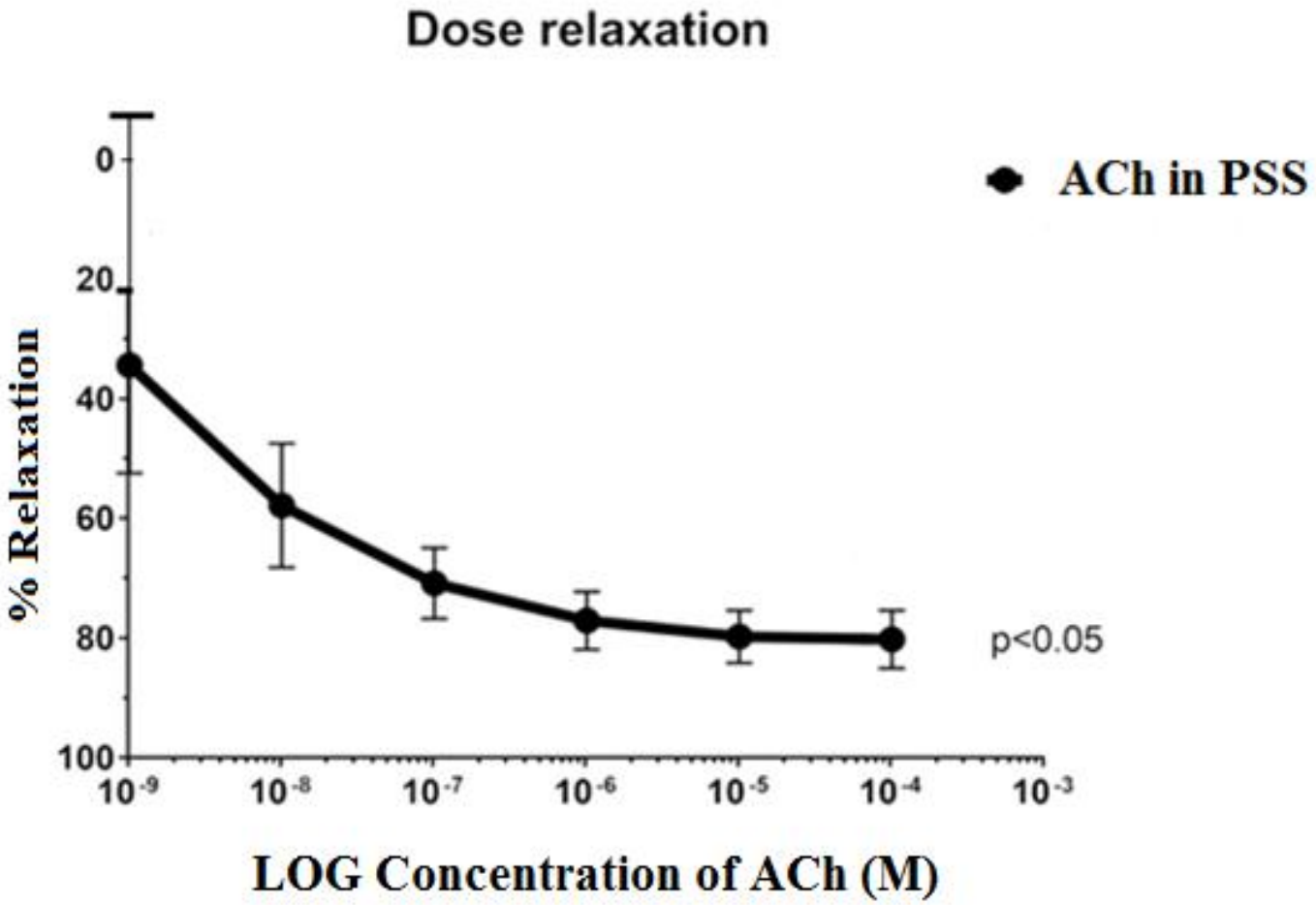


**Figure 1b**: shows a dose relaxation curve.

This figure represents the dose response to Acetylcholine.

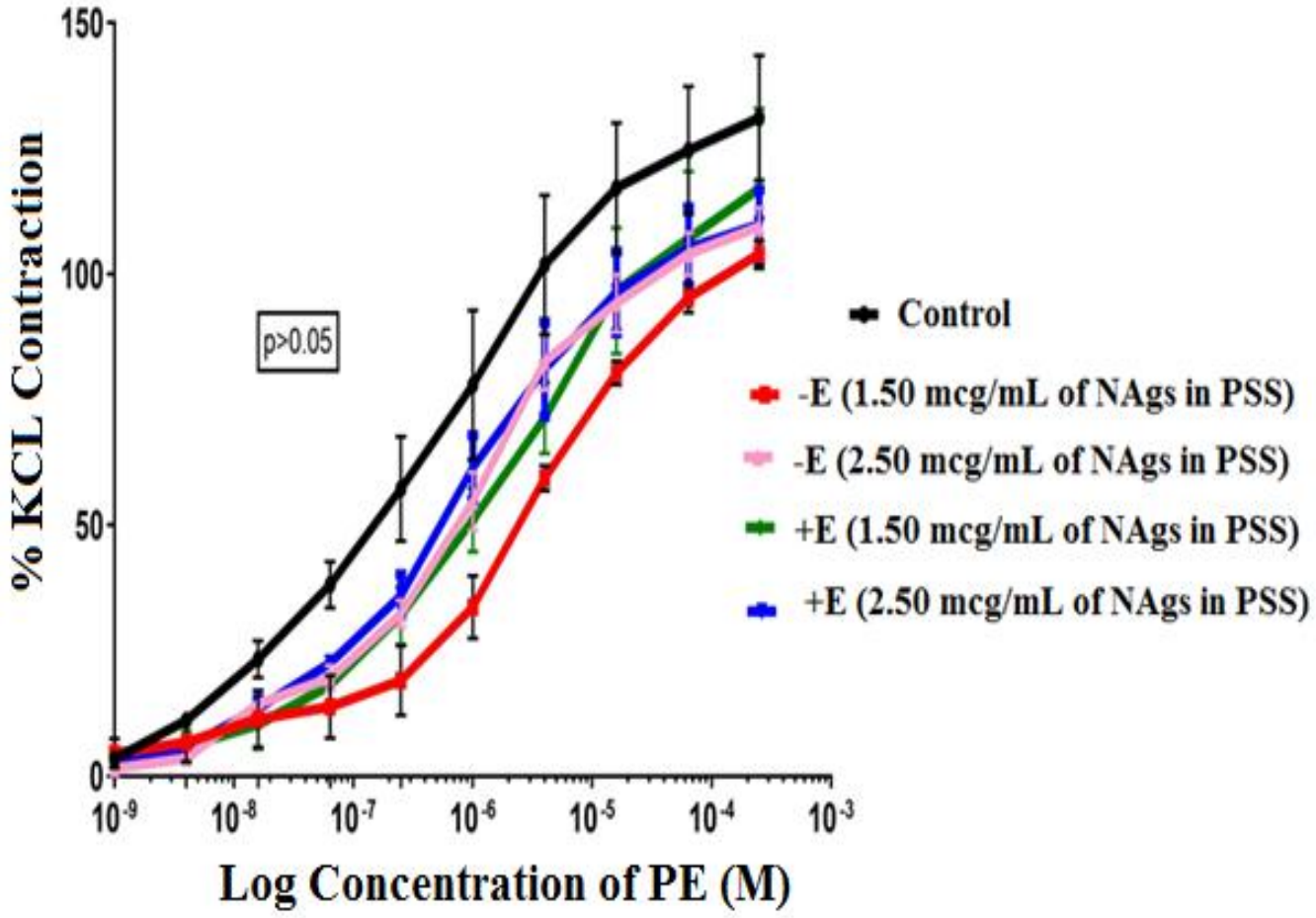


**Figure 2**: Shows the effect of nanosilver on contractile response induced by phenylephrine in varying concentrations of nanosilver solution in carotid arterial rings. n= 6, mean ± SEM. The graph shows a shift of the curve to the right showing nanosilver induced attenuated contractile response in varying concentrations in +E and –E carotid rings.

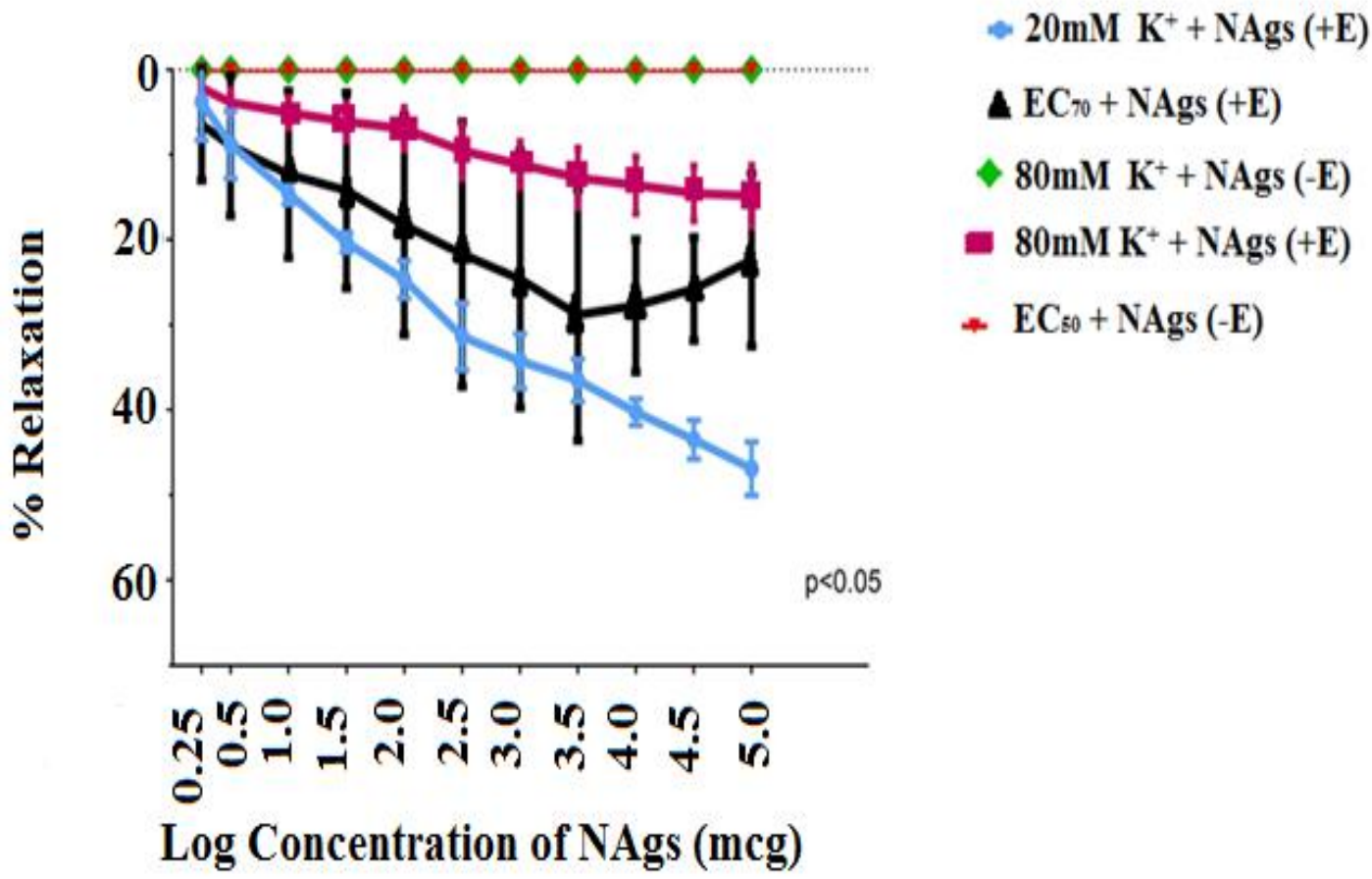


**Figure 3**: Showing the relaxation response induced by nanosilver solution following $EC_{70}$ M PE, (80 and /20) mM $K^+$ precontraction in +E and/or -E rings. N =6, mean ± SEM. Nanosilver induced relaxation is endothelium dependent

## ACh-induced relaxation in pretreated carotid arterial rings

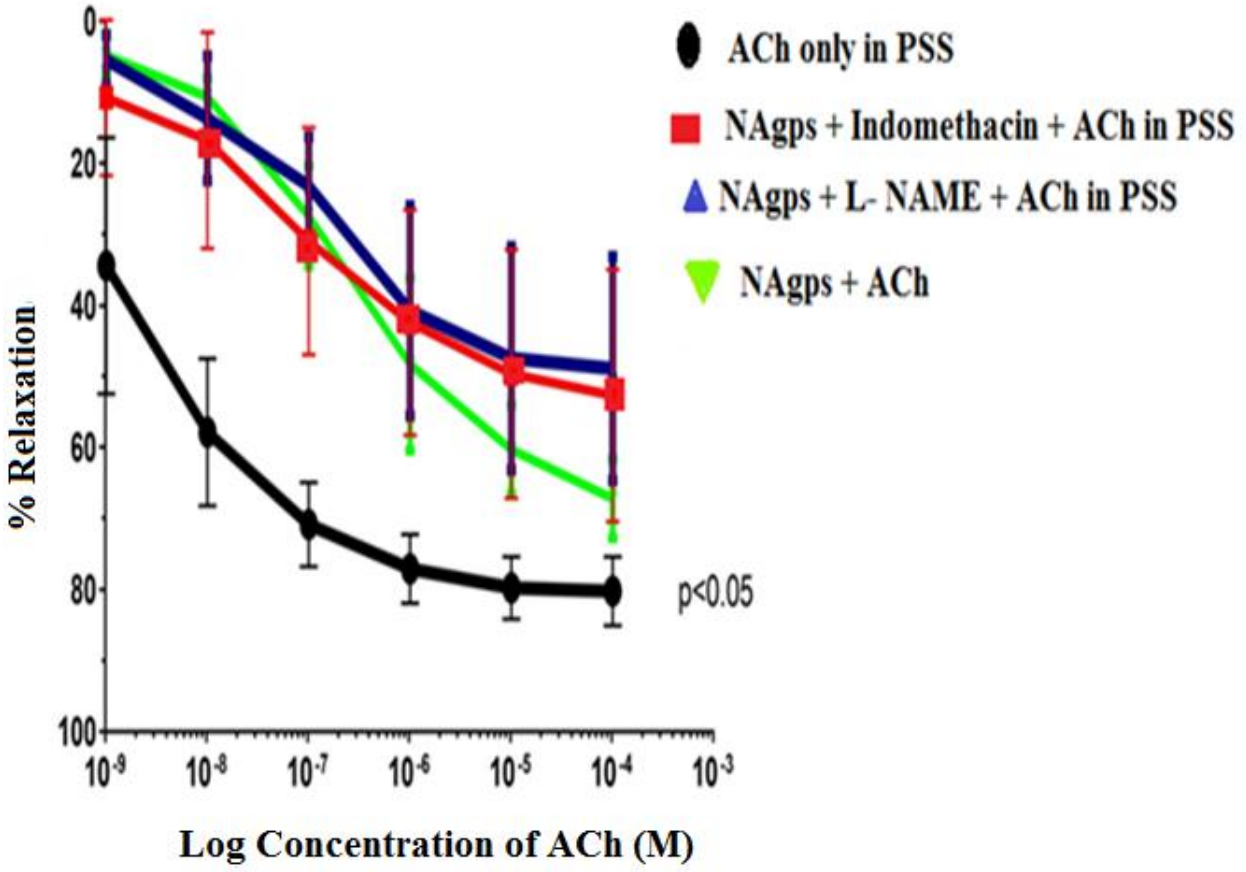


**Figure 4:** Showing ACh- induced dose relaxation response curves in normal PSS (control) and following exposures to L-NAME ($1x10^{-3}$) M or indomethacin ($3x10^{-6}$) M and/or nanosilver solution (state the concentration) PE precontracted carotid arterial rings. There was a shift of the test response curves to the right of the control showing attenuated relaxation and nanosilver endothelium non-specific mode of action. (N=6; means ± SEM).

**Phenylephrine $EC_{50}$ (M) contraction in varying NAgs concentration**

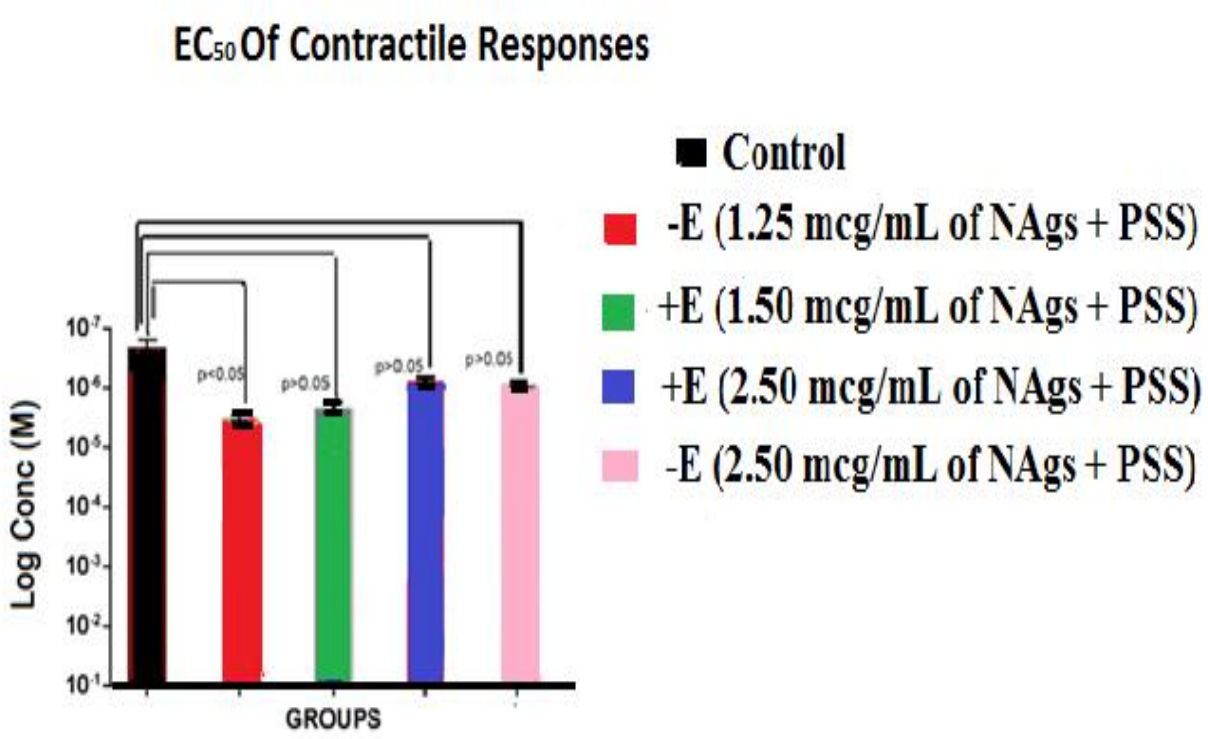


**Figure 5:** Comparative $EC_{50}$ (M) values of PE-induced contractions in +E and/or -E arterial rings exposed to varying concentrations of NAgs. (N=6; means ± SEM).

**Acetylcholine $IC_{50}$ (M) relaxation in NAgs relaxation**

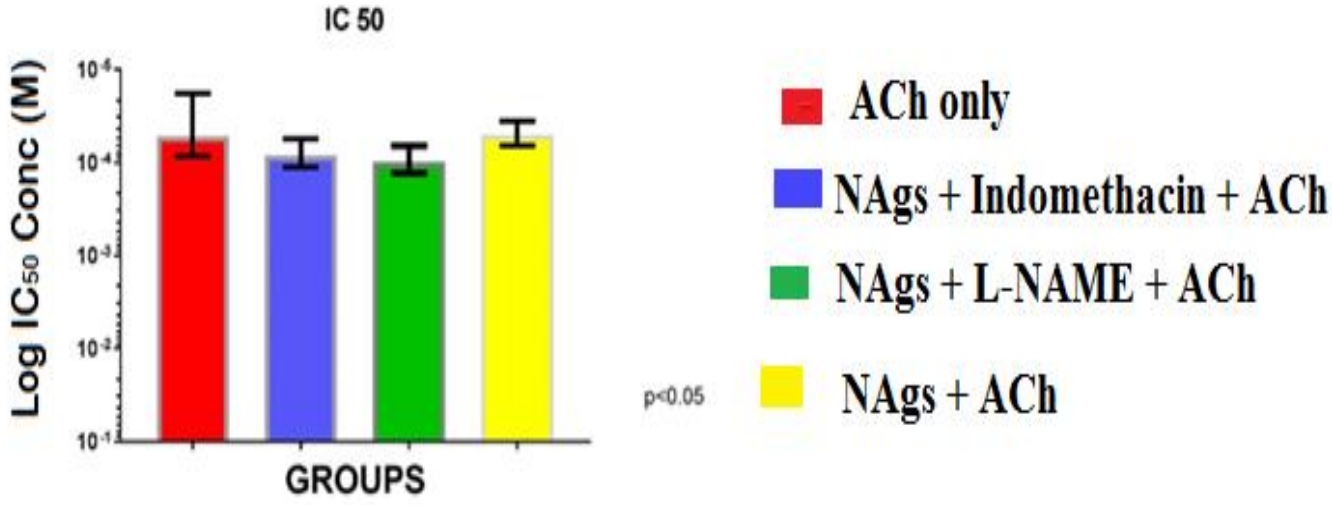


**Figure 6:** Comparative $IC_{50}$ (M) values of ACh-induced relaxation in PE-precontracted arterial rings exposed to varying agents. (N=6; means ± SEM).

**Nanosilver $IC_{50}$ (M) relaxation**

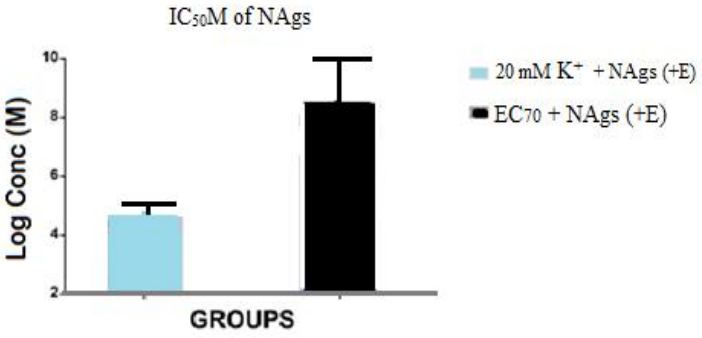


**Figure 7**

Showing the $IC_{50}M$ of NAgs-induced relaxation in Varying potassium depolarization. (N=6; means ± SEM).

# Chapter 5

## Discussion

This study investigates the effects of nanosilver on vascular reactivity in isolated rabbit carotid artery. The study showed the biphasic effect of nano silver, in which vasorelaxation was observed at low concentrations and vasocontraction observed at high concentrations in rabbit carotid artery. This study suggests that nanosilver induced relaxation is endothelium dependent.

To determine the contractile effects of Nano silver, we investigated the contraction induced by phenylephrine after exposure to nano silver, the result showed a shift of the dose response curve to the right showing nanosilver induced attenuated contractile response in varying concentrations in both +E and –E carotid rings. This result shows no significant effect of nanosilver to contractile responses.

To determine the contribution of EDHF or potassium channel in the effect of nano silver, carotid rings pre-contracted with 20mM $K^+$ showed significant relaxation to nanosilver. Studies have shown that the relaxation by nanosilver is due to the activation of endothelial nitric oxide synthase (eNOS), it was phosphorylated at serine residue 1177 (Rosas- Hernandez *et al*., 2009).

In an experiment to characterise nano silver mechanism of relaxation, carotid rings were exposed to nano silver for 20minutes before administration of Ach, NPs stimulated endothelial NO-dependent vasodilation in similar fashion to ACh, but in less magnitude.

In nano silver, L name and indomethacin pretreated rings there was a shift of the curve to the right showing attenuation of relaxation. The effects of Ach were abolished by pretreatment with a non-selective nitric oxide synthase (NOS) blocker, l-nitro methylarginine-ester (L-NAME), (Rosas-Hernandez *et al*. 2009).

Furthermore, our data shows that the biphasic, concentration-dependent effects of Nano silver is endothelium dependent. When the endothelium was removed from the rings, all physiologic responses were blocked. These results clearly demonstrate that the NPs have selective and specific effects on the vascular endothelium in a concentration-dependent manner and suggest that opposite effects could be associated with NPs of different sizes (Rosas- Hernandez *et al*., 2009).

The possible mechanism of action of nano silver was examined and the biphasic nature of nano silver was observed, in which vasorelaxation occurred at a low concentration and vasocontraction observed at a high concentration in rabbit carotid artery. This study suggest that the AgNPs may have dual effects on the vascular tone and may play a complex role in the selective and specific actions induced by AgNPs. Further research is required to fully understand the mechanisms of action (Gonzalez *et al.,* 2014).

These results clearly demonstrate that the selective and specific biological effects of nano silver on the vascular bed depend on the concentration of the nano silver and suggest that the opposite effects seen at high and low nano silver concentrations could be a result of nano silver size heterogeneity. Studies performed have provided evidence that suggest that Nano silver particles have dual and opposite effects on blood structure, angiogenesis and permeability and these effects depend on the size, surface chemistry, concentration of the Nano particles, target organ. In addition, the inadequate studies that have been performed in complex models of blood vessels and due to the fact that majority of them are concentrated on the angiogenic process prevents the creation of a definite

conclusion about the concern that Nanosilver particles are beneficial or harmful for human health. Moreover, the mechanisms of action of the biological interactions of nanosilver particles in the vascular system are still not well understood (Cui and Gao 2003; Rosas- Hernandez *et al*., 2009).

In spite of the many applications of NMs, many studies indicate that certain nano particles have controversial effects on human health due to the small size, reactive properties, and surface area of the particles. The small size and large surface area of NPs may allow them to enter and interact with cells and tissues. In the body, these NMs can translocate to sites distant from their site of entry (Nemmar *et al*., 2004; Oberdöster *et al*., 2005). Such translocation is facilitated by the propensity of NPs to enter cells, cross cell membranes, and be transported through the cardiovascular system, where the NPs interact directly with vascular endothelium and elicit several cardiovascular protective responses or causes disorders associated with inflammation (Yamawaki and Iwai, 2006; Yin *et al*., 2009).

## Conclusion

This study showed that NSPs causes relaxation of rabbit carotid artery.

NSPs induced relaxation is endothelium dependent.

Nanosilver has a biphasic effect on vascular tone in rabbit carotid artery when precontracted with $EC_{70}$.

Further studies should be carried out to fully understand the mechanism of action of NSPs and the adverse effects associated with NSPs.

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