EFFECT OF ADMINISTRATION OF SOMATOSTATIN ANALOGUE ON BLOOD PRESSURE IN CHRONIC INTERMITTENT HYPOXIC RATS

A thesis submitted in partial fulfillment
of the requirement for the degree of
Master of Science

By

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GRADUATE SCHOOL

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Kajal Kamra ENTITLED Effect of Administration of Somatostatin Analogue on Blood Pressure in Chronic Intermittent Hypoxic Rats BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF Master of Science.

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ABSTRACT

Kamra, Kajal, M.S. Department of Neuroscience, Cell Biology, and Physiology, Wright State University, 2019. Effect of Administration of Somatostatin Analogue on Blood Pressure in Chronic Intermittent Hypoxic Rats.

The cardiorespiratory system in our bodies does not adapt to Chronic Intermittent Hypoxia (CIH) and consequently syndromes such as sleep apnea lead to pathophysiological conditions like Hypertension. It has been demonstrated that the peripheral chemoreceptors underpin the development of these conditions and at present, there are no selective drug therapies for this form of hypertension. However, evidence suggests that peripheral chemoreflex sensitivity to CO_2 & hypoxia is reduced by Somatostatin (SST) in humans. Our preliminary in-vitro studies have demonstrated that SST will blunt the response of the carotid body to hypoxia and decrease the baseline activity of the carotid body. We therefore hypothesize that SST analogues given in-vivo via osmotic minipumps will attenuate the increase in BP, thereby preventing systemic hypertension from developing during CIH.

Adult male Sprague-Dawley rats were implanted with Osmotic minipumps containing sterile water or SST analogue (Octreotide Acetate) and then were exposed to CIH for ≤35 days to induce hypertension. BPs were measured before, during and after IH conditioning. Each minipump administers the drug for only 28 days. It was predicted that the animals exposed to the SST analogue will be protected from CIH-induced hypertension.

Data showed a sudden drop in BP post-surgery in all animals tested. This hypotension may have prevented the ability of chronic intermittent hypoxia to induce hypertension in both experimental and control animals.

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Please note: No editor was used in writing of this thesis.

INTRODUCTION

Anatomy and Physiology

The carotid bodies (also known as *glomus caroticum*) are small peripheral sensory organs located at the bifurcation of the common carotid artery which respond primarily to changes in blood oxygen levels, blood carbon dioxide levels or pH (Wyatt, Evans and Peer's review, 2010). The carotid bodies are also viewed as polymodal organ as they can respond to temperature and physiological fluctuations in extracellular potassium concentrations (Kumar, 2009) to produce cardio respiratory reflexes to restore blood gas homeostasis. The exact location of the carotid bodies is different among mammalian species and different individuals of the same species (Lopez-Barneo, Gonzalez, 2017) but is usually found at the bifurcation of the common carotid artery in the neck. The carotid body possesses of glomeruli / functional unit supplied with an extensive capillary network and connective tissue that separates glomus (T1) cells and sustentacular (T2) cells that are known to envelope type I cells (Lopez-Barneo, Gonzalez, 2017).

T1 (Glomus) cells are small (8-15 µm) ovoidal cells whereas, T2 (sustentacular) cells are elongated cell bodies (approximately 10 µm in diameter). Apart from their appearance, T1 and T2 cells can also be differentiated by abundance of mitochondria and presence of dense-core secretory vesicles in T1 cells (Lopez-Barneo, Gonzalez, 2017). The approximated ratio of T1:T2 cells is 4:1 (Donald et al., 1981). Other than T1 and T2 cells, CB of mammals also contain nestin^{+} cells that can produce fully grown glomus cells (Fig 1B &D**)** (Ortega-Saenz et al., 2013). Under hypoxia, T2 cells, which are known to be inactive stem cells can get activated and result in formation of T1 cells or other cell types (Pardal R et al., 2007).

Figure 1. A) Schematic from Peers, Wyatt, & Evans (2010) showing the gross morphology and anatomical location of the carotid body at the bifurcation of the common carotid artery (CC). The CC diverges into the internal carotid (IC) and external carotid (EC); at the base of this bifurcation the carotid body is normally found.

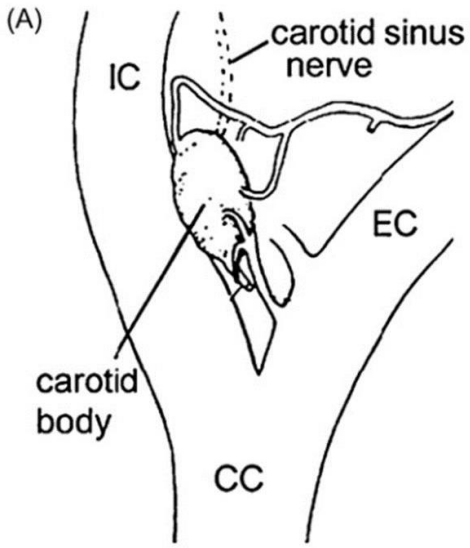
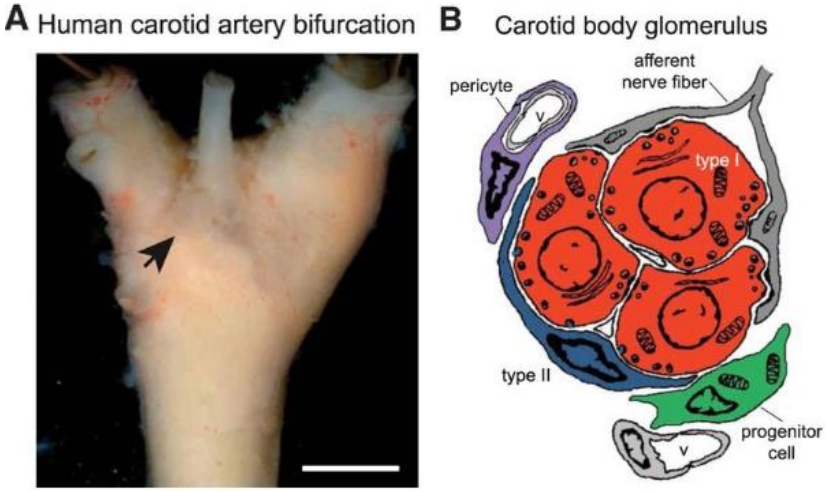
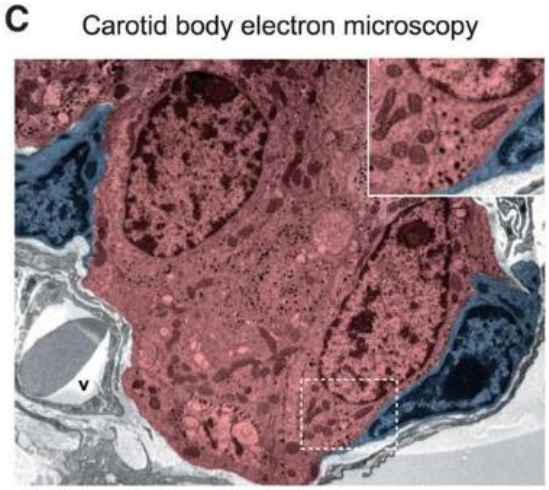
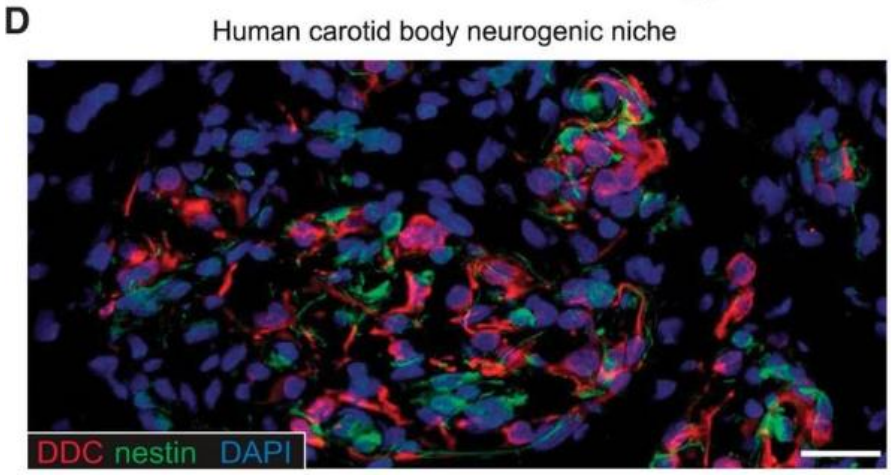

(A)
carotid sinus
nerve
IC
EC
carotid
body
CC

Figure 2. Structure and organization of the carotid body (CB) adapted from Jose Lopez-Barneo et al., 2016. **A)** Photograph of the human carotid bifurcation after cleaning the connective and fat tissue surrounding the CB (arrow) region. Scale bar, 1 cm. Male, 42 yr. old. **B)** Schematic representation of the cellular components in the CB glomerulus. v, Blood vessel. Nestin^{+} progenitor cells appear in green. **C)** Electron microscope photograph illustrating the ultrastructure and anatomical relations of the two main cellular elements in the mouse CB; type I cells (red) enveloped by type II cells (dark blue). The *inset* shows the abundance of mitochondria and dense core secretory vesicles in type I cells. **D)** Histological section of a human CB immunostained with antibodies against dopamine decarboxylase (DDC), a glomus cell marker, and against nestin, a progenitor cell marker. Scale bar 20 µm.


A Human carotid artery bifurcation
B Carotid body glomerulus
afferent
nerve fiber
pericyte
v
type I
type II
progenitor
cell
v


C Carotid body electron microscopy
v


D Human carotid body neurogenic niche
DDC nestin DAPI

In 1868, Pfluger announced that breathing is stimulated by hypoxia (Iturriaga, R., & Alcayaga, J. (2004)). In between 19^{th} and 20^{th} centuries, a structure similar to a "ganglion" (Iturriaga, R., & Alcayaga, J. (2004)) or "gland" (Nurse, C. A. (2010)) at bifurcation of common carotid artery was recognized by scientists but no physiological function was assigned to this organ until 1930. Heymans in 1930 recognized CB is a sensor of arterial hypoxia and was awarded with a Nobel prize for his contribution in this field. Verma et al., 1975 provided evidence that CB sensory activity disappeared when T1 cells were cryo destroyed. This identified the T1 cells as the chemosensors.

Neurotransmitters and their Release from Carotid Body Type I Cells

To counteract changes in blood gas or pH, T1 cells are activated and are depolarized by inhibition of K^+ channels which results in voltage gated Ca^{2+} entry and the neurotransmitter release. This then activates the afferent sensory nerve fibers of the CSN that along with CB T1 cells form a chemical synapse. Chemoreceptor information is then relayed to the respiratory center of brain stem to cause cardio respiratory and ventilatory reflexes restoring normal pH levels and blood gas levels in body (Ryan and Wyatt, 2018). CB T1 cells contain many neurotransmitters (mainly ACh, dopamine, ATP & Serotonin) of which ACh and ATP are likely to be accountable for activation of sensory nerve fibers (Fitzgerald et al., 1986; Zhang M et al., 2000)

Sleep Apnea, Hypertension and Intermittent Hypoxia

Sleep apnea is a clinically prevalent disorder in which breathing repeatedly stops and starts every 15-30 seconds. The main types of sleep apneas are Central Sleep Apnea (cessation of breathing of the respiratory rhythm) and Obstructive Sleep Apnea

(obstruction of the upper airway) (Nieto FJ et al., 2000; Young T et al., 1993.; Ramirez JM et al., 2013). The obstruction of the upper airway during sleep causes termination of air flow leading to intermittent hypoxia and affects an estimated 10 % of the population in the US (Nanduri, J. et al., 2017).

Patients suffering from SA can develop hypertension, myocardial ischemia, stroke and even unanticipated death (Hung et al., 1990). SA is a respiratory disease which can be caused by several factors. Apart from abnormal upper airway or defective central CO_2 chemoreceptors (Kumar, N. N. et al. 2015; Ramirez, J.-M. et al. 2013] it may also be caused due to abnormal CB activity (Orr, J. E., Malhotra, A., & Sands, S. A. (2017); Eckert, D. J., White, et al. 2013)**;** Eckert, D. J., Malhotra, A., & Jordan, A. S. (2009)).

Peng et al., 2017 proposed that CB chemoreflex activity may result in both Central and Obstructive Sleep Apnea. They also suggested that increased CB chemoreflex begins with carotid sinus nerve activity (CSA) which then lead to OSA by lowering the muscle tone of the tongue which lead to blockage of the upper airway.

Patients with OSA get mainly treated with Continuous Positive Airway Pressure (CPAP) which is known to improve oxygenation (Peng et al., 2017) but is not significantly effective in some patients, while some find it difficult to tolerate (Peng et al., 2017). An exaggerated CB chemosensory reflex in humans and rodents has been shown to contribute to IH-induced hypertension (Prabhakar et al., 2015).

By chronic ablation of CB or CSN that innervates the CB, chemosensory reflex gets disrupted, which prevents increased sympathetic nerve activity (SNA) as well as IH-induced hypertension in rats exposed to CIH (Fletcher et al., 1992**;** Peng et al., 2014).

Hypertension

"Population-based studies report an increased incidence of hypertension in OSA patients with strong correlation between the severity of apnea and hypertension." (Nandhuri et al, 2017).

When exposed to IH, rodents have been shown to mimic the blood O_2 saturation profiles during OSA and exhibit hypertension. (Fletcher, 1995; Kumar et al., 2006; Peng et al., 2006; Troncoso Brindeiro et al., 2007). The level of O_2 desaturation is parallel to elevation of systolic BP during SA which makes them hypertensive (Shephard et al., 1985). We know that intermittent hypoxia sensitizes the CB which increases SNA. A synergistic increase in SNA is seen when hypoxia and hypercapnia are the combined stimulus as seen in SA (Somers et al., 1988).

Considerable evidence suggests that cardiorespiratory systems can adjust to Chronic SH to enable to continue enough O_2 delivery to tissues (Lahiri, S., Rozanov, C. & Cherniack, N.S (2000); Bisguard, G.E. (2000). On the other hand, chronic IH causes pathophysiological conditions like hypertension, myocardial infarcts and even stroke (Nieto, F.J. et al.2000; Shahar, E et al. (2001)).

Apart from exposure to repetitive episodic hypoxia or stress due to normal sleep disruption, there are various mechanisms that could contribute to chronic increase in BP in OSA – Changes in salt and fluid balance (Krieger et al., 1991, Warley et al., 1988) and fluctuations in intrathoracic pressure that modifies the heart and vascular system have also been held responsible for BP changes in OSA (Bromberger-Barnea et al., 1981).

Evidence also suggests OSA as a self-sufficient hazard for systemic hypertension. It is known that OSA might lead to adverse cardiovascular consequences by increasing daytime and night time sympathoadrenal activity. Evidence shows elevated levels of urinary and catecholamine levels in OSA and increased muscle sympathetic nerve activity (SNA) during obstructive apnea. Hypertension of OSA is maintained even during wakefulness, thereby increasing risk of cardiovascular complications associated with hypertension (Sica et al., 2000).

Fletcher and his colleagues demonstrated that 35 days of episodic hypoxia mimicking the episodic hypoxemia of sleep apnea can cause a diurnal increase in mean blood pressure of 14 mmHg (Fletcher et al., 2017). The authors also reported that when rodents were exposed to episodic hypoxia, they exhibited an increase in Mean Arterial Blood Pressure (MAP). This increase was found to depend upon:

a) Peripheral afferent inputs transversing the CSN
b) Level of CO_2 in the hypoxic gas mixture; i.e., eucapnic hypoxia
c) An intact sympathetic NS (Fletcher et al.,1992).

Animals exposed to a similar experimental condition with a supply of room air for 30 days instead of N_2 showed change in MAP.

Fletcher and his colleagues did experiments focused on the roles of renal sympathetic nerve activity and the renin-angiotensin system in mediating the increased diurnal systemic hypertension in their model of IH (Fletcher et al., 2017). In their study, systemic hypertension was shown to be associated with activation of renin-angiotensin system as indicated by up-regulation of renin during the non-hypoxic periods of their protocol as well as the finding that blockade of the angiotensin II type I receptor eliminated the increase of

arterial blood pressure and renal nerve activity. These results provide substantial support for the role of the sympathetic system in the blood pressure response to long-term intermittent hypoxia (Fletcher et al., 1999). The key role of the sympathetic system was also supported by finding of A.L. Sica in anesthetized preparations that the amplitude of preganglionic SNA during hypoxia, hypercapnic, and asphyxic stimulation was significantly greater in animals previously exposed to CIH compared to control animals. The findings prove how chronic exposure to IH is linked with increase in activation of brain stem and some regions of neuro axis that are responsible in shaping the sympathetic outflow (Sica et al., 2000).

There is an increase in both SNA and minute ventilation from peripheral chemoreceptor stimulation in response to a hypoxic stress. It has also been shown that surgical removal of CB blocks sympathetic activation and prevents hypertension on IH subjected rats (Fletcher et al., 1992; Peng et al., 2003).

Somatostatin and its Analogue, Octreotide Acetate

After the discovery of SST in 1973 (Brazeau et al., 1973), a new approach to the treatment of conditions associated with endocrine hypersecretion, such as acromegaly, Cushing's disease, and symptoms of metastatic neuroendocrine tumors (NET), was initiated (Weckbecker et al., 2002).

SST was first described in the hypothalamus but SST producing cells are also found in central and peripheral nervous system, pancreas, gut, thyroid, adrenal, spleen, liver, kidneys and prostate. SST is also produced by immune cells. [Patel YC et al.,1999]. SST comes in 2 bioactive forms- SST 14 and SST 28 which are synthesized from

preprosomatostatin, a large precursor molecule SST is produced in different quantities by different cells and, at least in the rat, the gut accounts for 65% of total body SST-like immunoactivity, the brain for 25%, the pancreas for 5% and the remaining organs, for 5% [Patel YC et al.,1978]. SST is known to acts as a neurotransmitter and as an autocrine, paracrine or endocrine regulator. Numerous physiological functions including modulation of neurotransmission, cell secretion and proliferation, smooth muscle cell contractility, intestinal motility, absorption of nutrients and immune cell functions are controlled by SST (Patel YC et al., 1999).

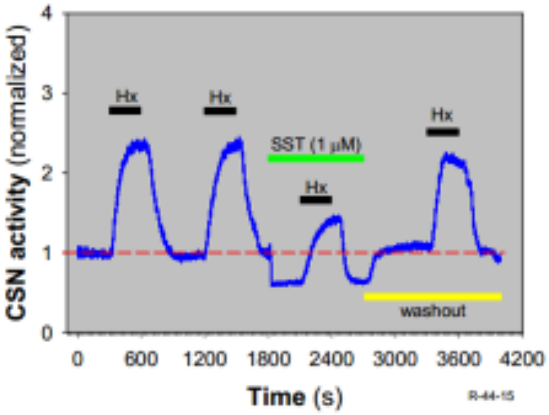
Effects of Somatostatin and the Acute Hypoxic Response

Somatostatin has been localized with in nerve fibers innervating the arterial chemoreceptors (Kummer et al., 1989, 1990 and Kameda 1989). In a small pilot study where Somatostatin Acetate dissolved in saline to achieve a concentration of 0.05 mg/ml was infused into a forearm vein in human subjects at the rate of 10 ml/h and lasted 64 minutes (Robbins et al., 1999). Their breathing, heart rate and blood pressures were recorded throughout the experiment. As a result, 5 out of 9 subjects reported side-affects like nausea, head ache and stomach-ache from SST administration. No changes in heart rate or blood pressures were found but SST attenuated the fast (peripheral) component of the ventilatory response to hypercapnia under hypoxia but had no effects on the slow (central) component of the ventilatory response to hypercapnia. It also depressed the ventilatory response to hypoxia (AVHR).

In 1995, e Silva & Lewis showed that somatostatin also inhibited whole cell calcium current peaks by 24% recorded from chemoreceptor Type I cells from the rat carotid body. *"In humans, infusions of somatostatin greatly reduce the ventilatory sensitivity to hypoxia (Maxwell et al., 1986 a, b; Filux et al., 1988). All this suggests that the most likely site of action of Somatostatin on the hypoxic ventilatory response is at the carotid body."* (Robbins et al., 1999).

Our collaborators, Dr. Richard Wilson and his students at University of Calgary, Canada carried out an *in-vitro* experiment where they took out rat carotid bodies still intact with the carotid sinus nerve (CSN) and perfused it with hypoxic perfusate. They saw an increase in the firing frequency along the sinus nerve. They then perfused it with SST (1µM) and saw a significant reduction in the baseline firing of the CSN activity. Upon washing the SST away, the CSN activity was restored to the normal levels (Figure 3). (UNPUBLISHED WORK)

Figure 3. Effect of SST on carotid sinus nerve *in-vitro* (Unpublished data from Dr. Richard Wilson's laboratory at University of Calgary, Canada): Upper figure- Y axis shows the change in CSN activity and the X axis shows the time in seconds. CSN activity increased when Carotid bodies (attached to CSN) were perfused with hypoxic perfusate (Hx) and the baseline CSN decreases when perfused with SST (1µM). The lower figure represents the mean data of this experiment in the form of bar graphs.


CSN activity (normalized)
Hx
Hx
SST (1 μM)
Hx
Hx
washout
0
600
1200
1800
2400
3000
3600
4200
Time (s)
R-44-15

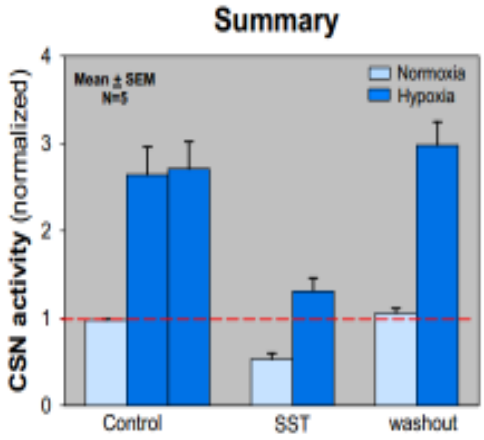

Summary
Mean ± SEM
N=5
Normoxia
Hypoxia
CSN activity (normalized)
Control
SST
washout

Table 1. Somatostatin and analogues: affinity for somatostatin receptors (Adapted from H. Reynaert et al., 2003).

		SSTR1	SSTR2	SSTR3	SSTR4	SSTR5	Adapted from Ref.
Endogenous SST	Somatostatin-14	+ +	+ +	+ +	+ +	+ +	20
	Somatostatin-28	+ +	+ +	+ +	+ +	+ +	20
Synthetic peptides	Octreotide	–	+ +	+	–	+ +	20
	Lanreotide	–	+ +	+ –	–	+ +	17
	Vapreotide	–	+	+	+ –	+ +	17
	Seglitide	–	+ +	+	–	+	20
	Somatuline	–	+ +	+	+	+ +	20
	SST-14-Dextran 70	+ +	+ +	+ +	+ +	+ +	19
Nonpeptide agonists	L-797,591	+ +	–	–	–	–	21
	L-779,976	–	+ +	–	–	–	21
	L-796,778	–	–	+ +	–	–	21
	L-803,087	–	–	–	+ +	–	21
	L-817,818	+	+ –	+ –	+ –	+ +	21
	KE108	+ +	+ +	+ +	+ +	+ +	22

Abbreviations used are as follows: SST: somatostatin; SSTR: somatostatin receptor subtype.

SST and its analogues work through six G-Protein coupled receptors (SST1 through 5) including two splice variants, SST 2A and SST 2B (Goodchild AK et al., 1982**;** Padley JR et al., 2003) that control the activity of ion channels ($Ca2^{+}$, K^{+}, Na^{+} and Cl^{-}) and enzymes (Adenyl Cyclase, Phospholipase C, Phospholipase A2, Phosphoinositide 3-Kinase and Guanylate Cyclase) culpable for the synthesis or degradation of intracellular second messenger (CAMP, inositol 1,4,5-triphosphate, diacylglycerol and CGMP). All the SSTRs are coupled negatively to adenylate cyclase, activate phosphotyrosine phosphates, and modulate MAP kinase, whereas subtype-specific actions are also evident (Goodchild AK et al., 1982; Padley JR et al., 2003).

There is evidence that SSTR and SST containing cell bodies and terminals are present in the ventral medulla where the respiratory centers are located (Peter G.R. Burke

et al., 2008). Not much is known about their functional roles, except that microinjection of SST evokes apnea (Peter G.R. Burke et al., 2008).

Peter G.R. Burke found that when SST is bilaterally injected at Rostral Ventrolateral Medulla (RVLM) pressor sites, it evokes bradycardia, a large depressor response and robust sympathoinhibition. They also found that activation of SST2A receptors in RVLM, located on only approximately 35% of pre-sympathetic neurons containing TH and/or PPe, evoked intense sympathetically mediated hypotension and bradycardia.

SST is known to attenuate cellular proliferation by a direct effect on cellular division (Payan D et al., 1984), by reduction of the release of both Growth hormone (GH) (Moreau et al., 1987) and Insulin Growth Factor (IGF) (Grant MB et al.,1994) and reduction of Ca2+ currents in neuron (Wang H et al., 1990). Tjen-A-Looi S in 1992 provided evidence that chronic infusion of SST-14 neurons (20ug/rat/h) reduces pulmonary arterial pressure in chronically hypoxic rats.

SST analogues are efficiently linked to the human SSTR selectivity profile, with a synergistic SSTR2 and SSTR5 binding appearing critical (Tolis et al., 2006). One such synthetic analogue of SST, Octreotide (an eight amino acid cyclic peptide analogue) has been in clinical trials since 1984. It has more protracted pharmacological actions than SST and primarily bind with high affinity to human SSTR2 but show reduced or no binding to SSTR 1, 3, 4 and 5 (Table 1) (Tolis et al., 2006). In many studies, Octreotide has been used to treat acromegaly and esophageal variceal hemorrhage in humans mainly as bolus injections of 50µg followed by 25-50µg/h (Reynaert et al., 2003). Some also use Long Acting Release (LAR) Octreotide by deep subcutaneous depot injections to administer a dose of 20mg-40mg/month (Colao et al., 2009; Tolis et al., 2006).

Patients with acromegaly tend to develop hypertension due to multifactorial mechanisms. In a published study by George Toils and others in 2006, when treated with Octreotide for six months, there was a decrease in systolic blood pressure in patients with left ventricular hypertrophy.

"SST analogues (SSTA) have a beneficial effect on BP (G. Minniti et al., 2001; A. Coloa et al., 2001), significantly improve heart rate and cardiac function, reduce left ventricular mass, improve systolic and diastolic function, and exercise training (G. Minniti et al., 2001; A. Coloa et al., 2000; P. Maison et al., 2007), improve diastolic filling (L. De Marinis et al., 2008), and may even reduce the rate of arrythmias (A. Calao et al., 2012; G. Lombardi et al., 2002), without a significant influence over heart valve disease (A.A. van der Klaauw et al., 2006; A.A. van der Klaauw et al., 2008)."

In another study, eleven patients (seven females and four males) received Octreotide (100-200µg), three times daily, subcutaneously over a 6-month period. Overall, systolic and diastolic mean pressures, heart rate and electrocardiograms did not change significantly throughout the investigational period (Bartolomeo Merola et al., 1993). There is some evidence that SST can modify the renin-angiotensin system in a manner that could lower BP independent of any effect on the endocrine pancreas (Izumi Y et al., 1980).

Hypothesis and Summary

We hypothesized that SST analogues given *in-vivo* will attenuate systemic hypertension from developing during intermittent hypoxia by attenuating an increase in BP.

To test this hypothesis, we used 32 male Sprague Dawley rats – control rats (n=16) and experimental rats (n=16). The experimental rats were exposed to Chronic Intermittent Hypoxia (4-5% O2, 15 events/hour, 8 hours/day for ≤35 days) to induce hypertension. The control rats were exposed to room air throughout the experiment. ALZET 2ml osmotic minipumps were surgically implanted subcutaneously to administer SST analogue, Octreotide Acetate in rats. Blood pressure was recorded before and after the surgery and weekly thereafter by using CODA non-invasive BP system.

MATERIALS AND METHODS

Animals

38 adult male Sprague Dawley rats (170g) were used in this experiment (Envigo, Indianapolis, IN) They were randomly subcategorized into control (n=19) and experimental (n=19) groups. They were weighed separately at the time of arrival and each week thereafter. They were all fed standard rat chow (Teklad 8640 from Envigo) and water.

Standardized Blood Pressures for Rats

From day 4 to day 5, i.e. after 3 days of rest and acclimation to the room, the rats were acclimated to the CODA non-invasive blood pressure system (a tail-cuff method, Kent Scientific Corporation) for 20 cycles per rat between 8:30 am to 11am only. On day 6, the day before surgery, each animal was weighed, and baseline BPs were recorded using the CODA noninvasive BP system. BPs were also recorded two days after surgery (Post-op BP) (day 9), on first (day 16), second (day 23), third (day 30) and fourth (day 37) week of exposure to CIH.

After placing the rats in the CODA rat holders/tubes (Figure 4) (made sure they were comfortable and not squished), the holders were placed on top of a dry paper towel on the warming platform/tray (to the desired temperature, 34^0C). The O-cuff and VPR-cuff were gently placed up the tail until resistance was felt. A slight gap, not more than 2 mm was maintained between the two cuffs. These tail cuffs were attached to the CODA machine which was attached to a computer (Figure 5) where all the recordings were recorded.

For at least 10 minutes, the rats could get acclimated and the tail temperature to rise to 34^0C. To achieve adequate tail temperatures quickly, a warm water filled glove was placed on the rat's tail (avoiding tubing) and a warm pad (Kent Scientific) over the restraint

for rats. Water-glove and heating pad were placed and removed as necessary to maintain proper tail temperature.

Once the rats were calm & up to temperature, we ran the cycles. Each rat ran 1 time with a total of 5 acclimation and 15 regular cycles. The data was exported and saved by date, group and animal number onto a MS Excel spreadsheet for further statistics. No CIH exposure on the day of BP measurement was done.

Figure 4. A) Image of Rat Holders in-use. (Adapted from Kent Scientific Corporation Website- https://www.kentscientific.com/products/productView.asp?productID=6376&Mouse_Rat=Holders&products=Nose+Cone+Animal+Holders+with+Stand) **B)** Image of warming pads used to keep the rat restrainers warm. (Adapted from Kent Scientific Corporation website- https://www.kentscientific.com/products/productView.asp?productID=6234&Mouse_Rat=Warming+Pads&products=Far+Infrared+Warming+Pad)

A)

B)

Figure 5. Schematic of Measurement of Blood Pressure using CODA non-invasive tail cuff method.

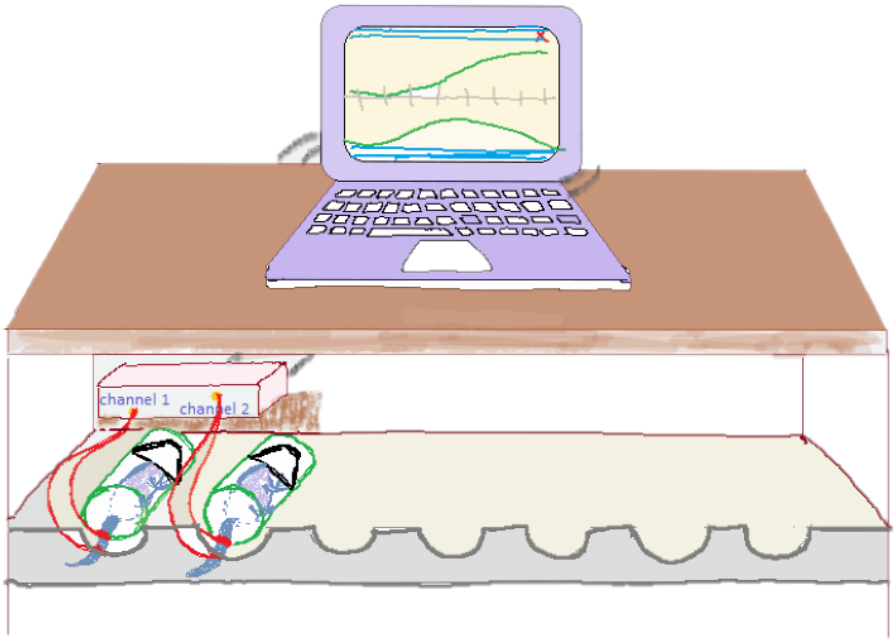
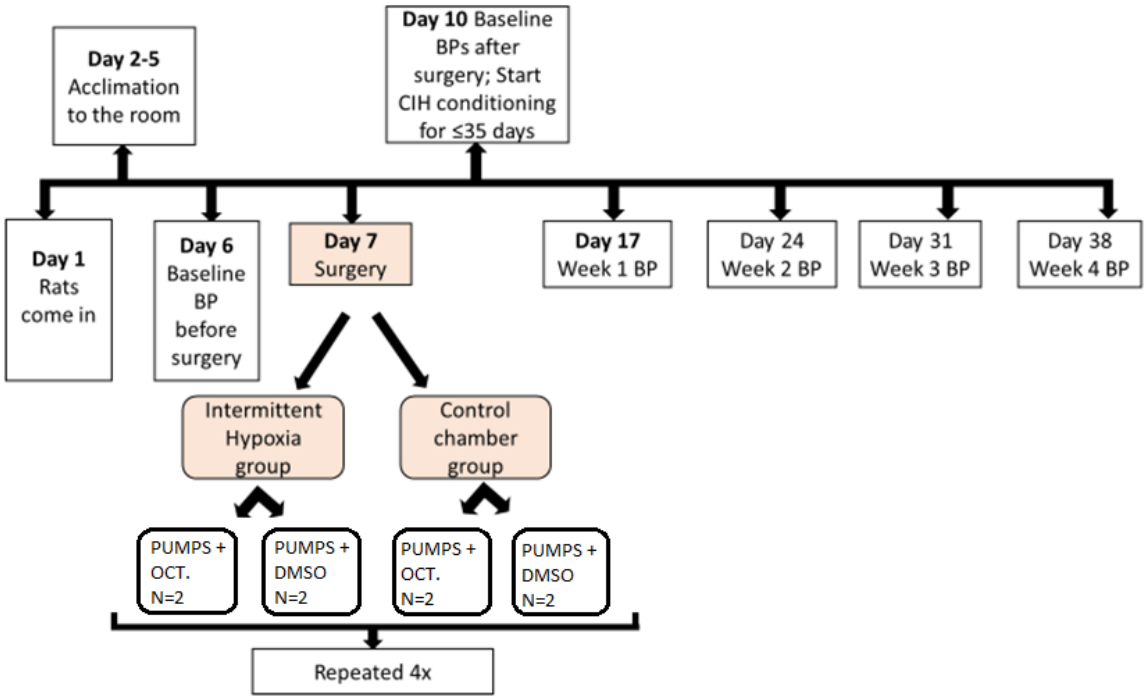

channel 1
channel 2

Figure 6. Schematic of experimental timeline used in this research project. Animals were acclimated to the experimental room for 3 days. On day 7, ALZET 2ml4 osmotic minipumps containing either DMSO or drug were surgically implanted subcutaneously in the back of the animals and blood pressure was recorded before and after the surgery and weekly thereafter for 4 weeks.


Day 2-5
Acclimation to the room
Day 10 Baseline BPs after surgery; Start CIH conditioning for ≤35 days
Day 1
Rats come in
Day 6
Baseline BP before surgery
Day 7
Surgery
Day 17
Week 1 BP
Day 24
Week 2 BP
Day 31
Week 3 BP
Day 38
Week 4 BP
Intermittent Hypoxia group
Control chamber group
PUMPS + OCT. N=2
PUMPS + DMSO N=2
PUMPS + OCT. N=2
PUMPS + DMSO N=2
Repeated 4x

Surgery

On day 7, we surgically implanted ALZET 2ml4 osmotic mini pumps (Model 2ML4; Alzet Corp., Palo Alto, CA) that were filled with 8.5µg/ml/Kg of Octreotide Acetate (Somatostatin Analogue from Fisher Scientific)/Sterilized Water for the experimental/ control animals respectively (The standard protocol uses 10µg/ml/Kg of Octreotide per rat (Schmid et al., 2012)). Each animal weighed different at the time of surgery, so different concentrations of drug were calculated accordingly.

On each experimental day, eight male Sprague Dawley rats (45-60 days old), one at a time, were exposed to 2-5% isoflurane and 100% oxygen at a flowrate of 0.5L/min in an anesthesia induction chamber. Once the animal was unconscious, it was transferred to a nose cone underneath a dissecting microscope where it was exposed to the same anesthetic gas and flow rate as in induction chamber. Reaching the proper anesthetic plane was ensured by testing the animals' withdrawal reflex using a foot and tail pinch. Once the rat had no response to the pinch, its limbs were taped down to keep it stationary, and the pump implantation surgery was carried out.

Each implantation started with the shaving of the incision area with an electric razor and cleansing with 70% Ethanol; followed by scrubbing the area thrice with betadine using cotton applicators (Fisher Scientific) and cleansing with 2" x 2" cotton gauze pad (Fisher Scientific) and then again with 70% ethanol & gauze pad.

Using a surgical scalpel (World Precision Instruments, Scalpel Handle #3, German 500236-G), a small mid-scapular incision was made along the vertebral column to expose subcutaneous fascia. Then by using a bigger surgical scissor and a needle holder (Fine

Science Tools, Crile-Wood, 12503-15), this initial incision was enlarged in order to make a pouch/pocket subcutaneously to hold the sterile osmotic mini pump. The sterilized osmotic mini pump was then placed longitudinally (parallel to the spinal cord): flow moderator facing posteriorly (facing towards the tail) within the subcutaneous cavity. Any excessive bleeding was wiped off with a 2" x 2" gauze. To close the incision site, VISORB (absorbable) sutures were used (CP Medical, Coated Visorb, violet braided, Size: 4-0, Needle:C-3, Shape: 3/8 Circle). Starting with a surgeon's knot on the posterior end of the incision and followed by continuous stitch on the way up to the top of the incision and then closing it up with another surgeon's knot. The incision area was again lightly scrubbed with betadine to kill any bacteria or germs in that area to prevent infection. Immediately after the completion of each survival surgery, the rats were given a 0.1ml/100g dose of Carprofen as a pain reliever.

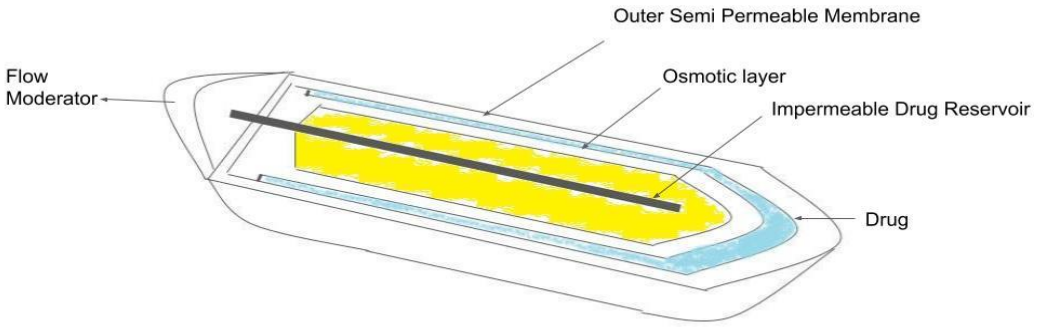
Working of ALZET 2ML4 Osmotic Mini Pump

Osmotic pump is made up of an outer semi permeable membrane, middle osmotic layer and an inner impermeable drug reservoir of 2 ml (2000 µl). The pump is designed to administer the drug at a pump rate of 2.5µl/h continuously for 28 days when filled and implanted. The fill volume of the pump reservoir should be over 90% of the mean fill volume (i.e. equal to 2000 µl) indicated in the lot specifications at the top of the instructions sheet in the ALZET 2ML4 osmotic mini pump box.

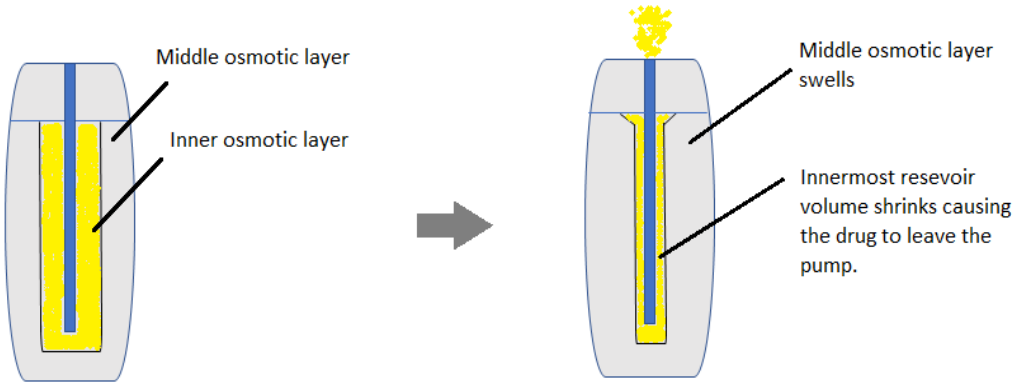
To avoid puncturing the pump reservoir, the filling needle provided with the pumps was only used. The pump was then capped with the flow moderator.

Once implanted, body fluid from the animal is taken up by the outermost semipermeable membrane, followed by the middle osmotic layer of the pump causing it to swell and thus shrinking the innermost reservoir volume and causing the drug to be delivered out of the pump into the animal's body via flow moderator.

Figure 7. Schematic of ALZET osmotic minipump.



ALZET 2MI4 OSMOTIC MINI PUMP



Exposure to Chronic Intermittent Hypoxia (CIH)

Freely mobile animals housed in a feeding cage (11 x 11.5 x 17 inch), two cages per session with up to four animals per cage were placed in hermetically sealed chronic intermittent hypoxic chambers (Figure 8) (15 x 20 x 21 inch; COY). With the help of gas mixer, the hypoxic chamber was flushed with 100% Nitrogen for 36 seconds to reduce the ambient fractional concentration of Oxygen (FIO_2) to 5% for 1 sec. This was followed by infusion of Oxygen allowing FIO_2 = 21%. In the meanwhile, when Nitrogen was being supplied to the hypoxic chamber, normoxic chamber was supplied with compressed air to simulate the same noise and air disturbance. The cycle was repeated 15 times/hour for 8hours/day for $\leq$ 35 days.

This entire set of experiment was repeated with four different times, each time with a new set of animals (therefore, total N=32). Paired student's T-Test was run for each group of animals and values ≤ 0.05 were considered statistically significant. Michael Bottomley, Statistical Programmer Analyst at Wright State University did a separate analysis for the data obtained through this experiment. For each of the three response variables (SBP, DBP and MAP), a repeated measures mixed effects ANOVA with a random effect for rat ID was run. The repeated measures aspect takes the correlation between time points for each rat into effect and the random effect factors in the correlation for the 5 measurements on each rat at each of the time points. A level of significance of alpha = 0.05 was used for each model. (See Appendix)

Figure 8. Experimental setup used to condition animals to Chronic Intermittent Hypoxia.

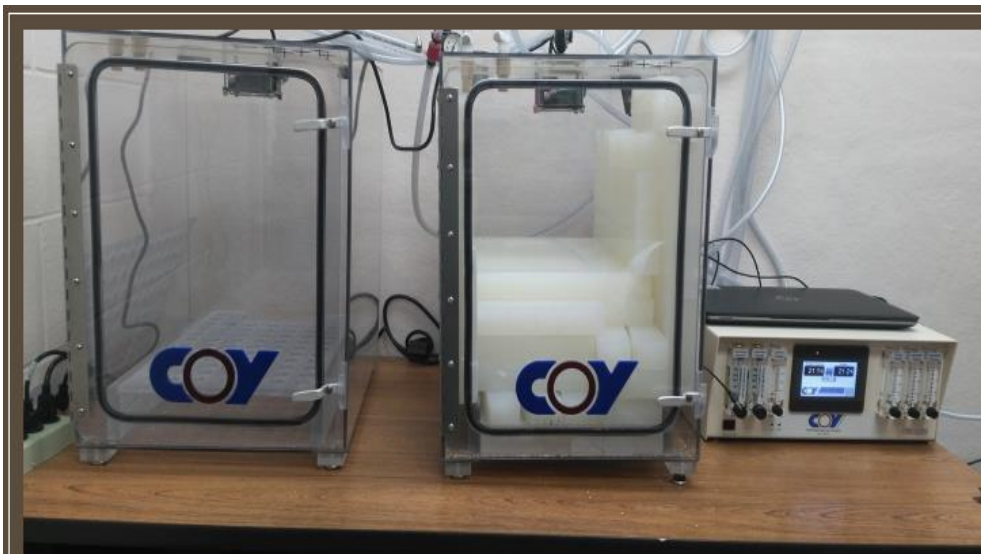
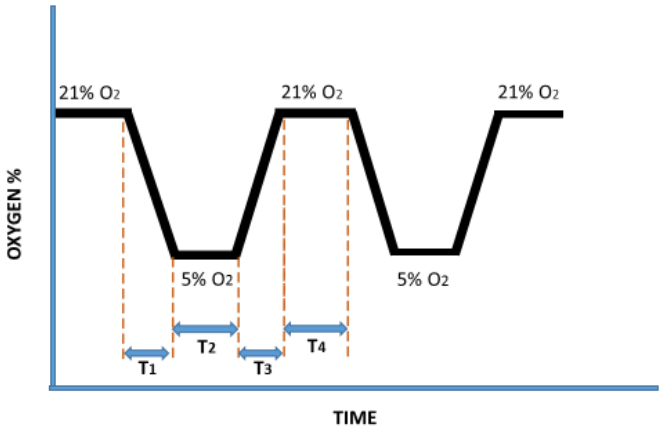

COY
COY

Figure 9. Chronic intermittent hypoxia system kinetics. T_1: ramp down to hypoxia, T_2: hypoxia soak, T_3: ramp up to normoxia, T_4: normoxia soak



T1 = 36 sec
T2 = 1 sec
T3 = 34 sec
T4 = 2 min 50 sec

RESULTS

Standardized Blood Pressures for Rats

This study is done to test the hypothesis that a sustained allocation of SST or SST analogue will attenuate BP in chronic intermittent hypoxic rats. Thirty-two male Sprague Dawley rats separated into two equal groups- Control (CNTRL) and Experimental (CIH) (n=18), were further divided into four equal sub-groups – Control + No Drug (CNTRL + DMSO), Control + Drug (CNTRL + SST), Experimental + No Drug (CIH + DMSO) and Experimental + Drug (CIH + SST). After three to four days of acclimation to the room, baseline blood pressures (Systolic, Diastolic and Mean arterial) were measured one day before the surgery (PRE) and then on the third day after surgery (POST) and then weekly thereafter for up to four weeks (W1→ W4).

Figure 10 shows change in SBP from week 3 to week 4 in all 6 groups of animals. On Y axis is the systolic BP and on X axis are CNTRL + DMSO, CNTRL + SST, CIH + DMSO, CIH + SST, CNTRL group without surgery and CIH group without surgery. Error bars are the standard errors of the mean calculated as standard deviation/square root (n-1). A significant drop in SBP is seen from week 3 to week 4 in CNTRL + SST group, which opposes our hypothesis as this group was not expected to show any significant change in BP. A significant decrease ($p \leq 0.05$) (student's t-test) in SBP is seen from week 3 to week 4 in CIH + DMSO group of animals. This group also opposes our hypothesis. CIH + SST group of animals do not show a significant change in BP. This group was expected to show a significant decrease in BP at the end of week 4 and therefore failed our hypothesis.

The last two groups depict results as expected on exposure to normoxia and CIH (but without surgery). A significant increase in BP is seen in animals that were exposed to CIH in comparison to animals that were not. This ensures that the CIH system works effectively.

Figure 10. Comparison of results of animals that did not undergo pump implantation (surgery) with animals that did undergo surgery for pump implantation.

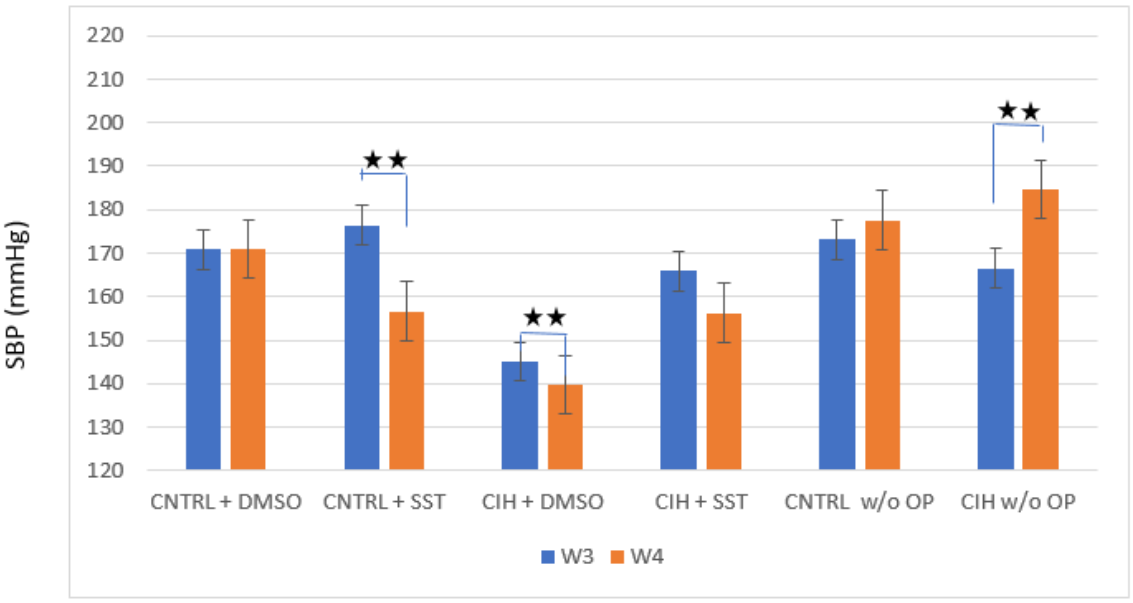

SBP (mmHg)
220
210
200
190
180
170
160
150
140
130
120
★★
★★
★★
CNTRL + DMSO
CNTRL + SST
CIH + DMSO
CIH + SST
CNTRL w/o OP
CIH w/o OP
W3
W4

Figure 11 shows the variability of the method measuring BP by tail cuff plethysmography. Y axis is the Systolic BP (mmHg) and on the X axis are 6 random animals from CNTRL + DMSO group. For each animal 5 SBP recordings are taken into consideration and variability with in each animal as well as between animals is depicted in the following figure.

For animal 1, four out of five recordings fall in the range of 165mmHg -190mmHg. For animal 2, SBP recordings are in the range of 155mmHg-175mmHg. Animal 3 SBP is the range of 177mmHg-198mmHg. Animal 4 is in the range of 154mmHg-165mmHg. Animal 5 is in the range of 190mmHg-215mmHg. Animal 6 shows most variation with in itself with 3 readings being in the range of 129mmHg-145mmHg and other two in the range of 173mmHg-183mmHg.

All these SBP ranges are higher than the normal SBP range which is 120-140mmHg.

Figure 11. Variability of tail-cuff BP measuring system with in animal and among other animals of CNTRL + DMSO group.

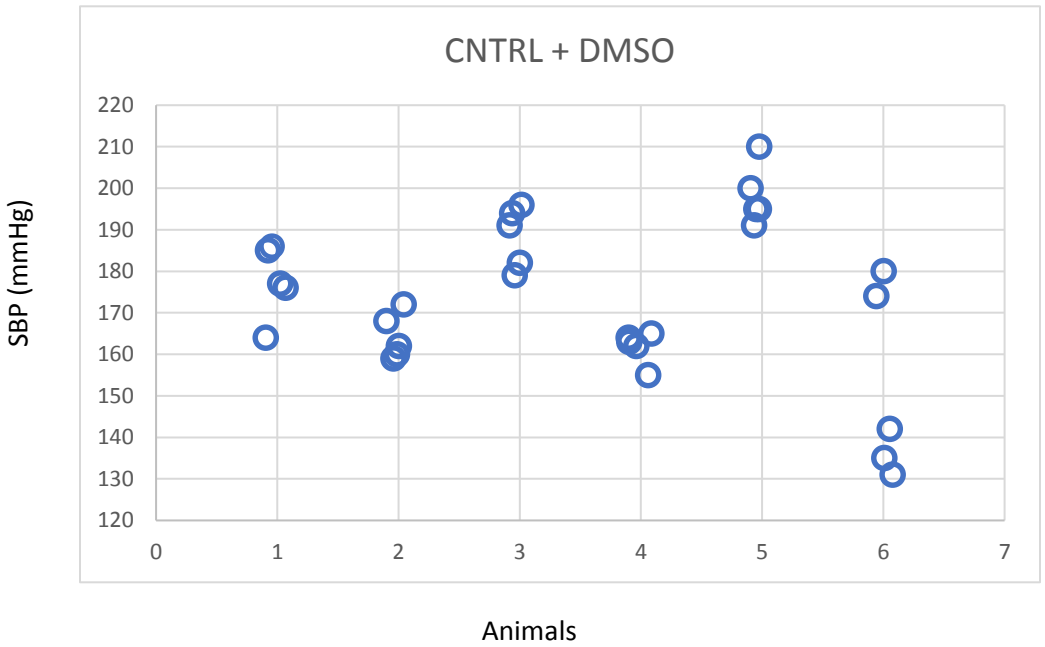

CNTRL + DMSO
SBP (mmHg)
220
210
200
190
180
170
160
150
140
130
120
0
1
2
3
4
5
6
7
Animals

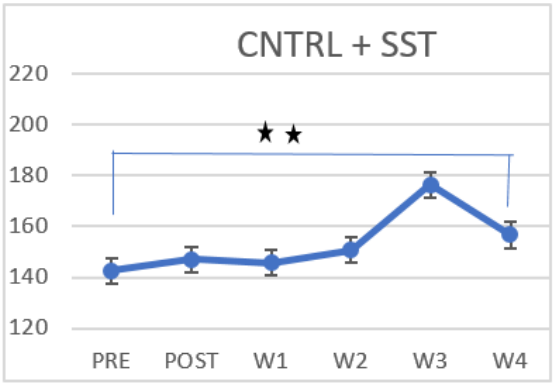
Figure 12 shows changes in SBP throughout the experimental timeline in CNTRL + SST (n=8), CNTRL + DMSO (n=8), CIH + SST (n=8) and CIH + DMSO (n=8) group of animals. Y axis is the SBP and on the X axis is different timepoints at which BP was measured- pre-surgery, post-surgery, week 1, week 2, week 3 and week 4. Error bars are the standard error of mean calculated as standard deviation/square root(n-1).

For CNTRL + DMSO group, no significant difference ($p>0.05$) is seen from post-surgery to week 4 hence, supports our hypothesis. In CNTRL + SST group a significant increase is seen from post-surgery to Week 4 BP. In CIH + DMSO group, a significant increase ($p\leq 0.05$) is seen from pre-surgery to post-surgery in systolic blood pressure, no significant change in BP is seen from pre-surgery to post-surgery BP for CIH + SST group of animals. A significant increase ($p\leq 0.05$) is seen from post-surgery to week 4 in DMSO containing CIH exposed group, but no significant difference is seen in case of drug containing CIH exposed group of animals. CIH + DMSO group of animals oppose our hypothesis. They should have had an increase in BP in comparison to CIH + SST group. CIH + SST also failed the hypothesis as it shows an increase in BP. All groups showed a drop in BP at week 1. This could be because some other mechanism in the body tries to bring the BP to the normal range to maintain homeostasis.

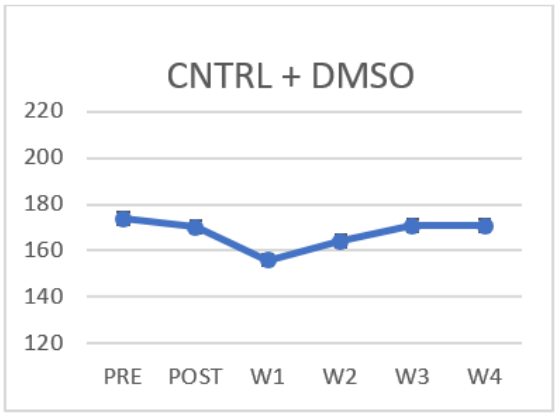
Figure 12. Comparison of four groups of animals studied in this experiment showing that surgery/implantation of osmotic minipump causes a consistent, but transient, drop in BP.

A) CNTRL + DMSO, B) CNTRL + SST, C) CIH + DMSO and D) CIH + SST

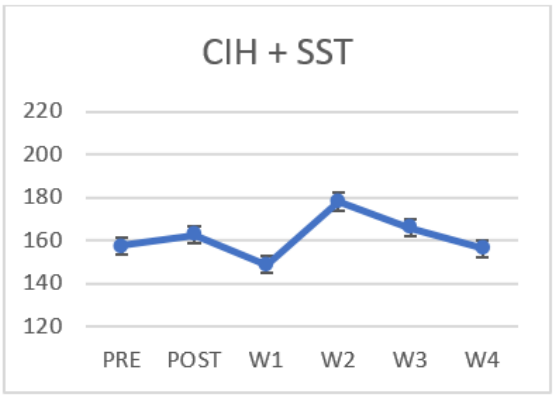
A)


CNTRL + SST
220
200
180
160
140
120
★ ★
PRE
POST
W1
W2
W3
W4

B)


CNTRL + DMSO
220
200
180
160
140
120
PRE
POST
W1
W2
W3
W4

C)


CIH + SST
220
200
180
160
140
120
PRE
POST
W1
W2
W3
W4

D)

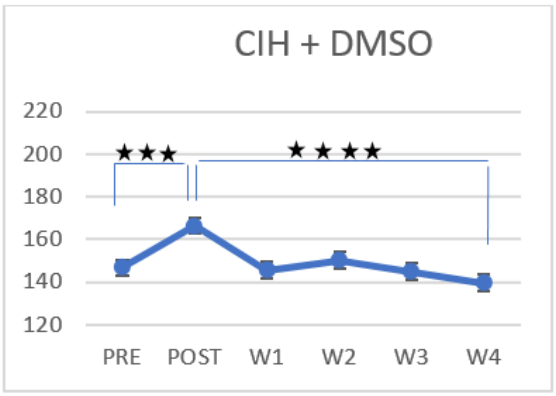
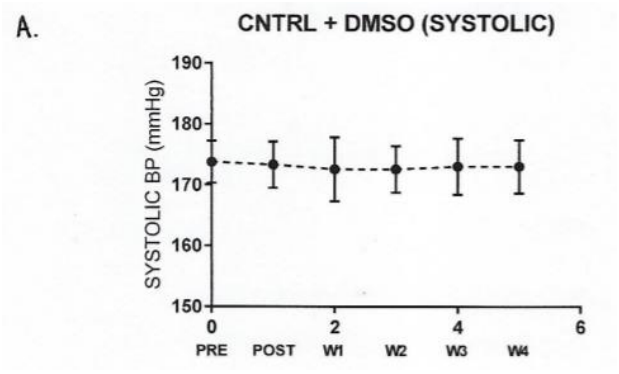

CIH + DMSO
220
200
180
160
140
120
★★★
★★★★
PRE
POST
W1
W2
W3
W4

Figure 13 shows the predicted effects of DMSO in sham control group (n=4) on systolic, diastolic and mean arterial BP. Figure A shows systolic BP (mmHg) on Y axis and pre-surgery BP, post-surgery BP, W1 BP, W2 BP, W3 BP and W4 BP on X axis. Same axis is followed for figure B Diastolic BP and figure C- MAP. Error bars are standard error of the mean calculated as standard deviation/ $\sqrt{(n)}$.

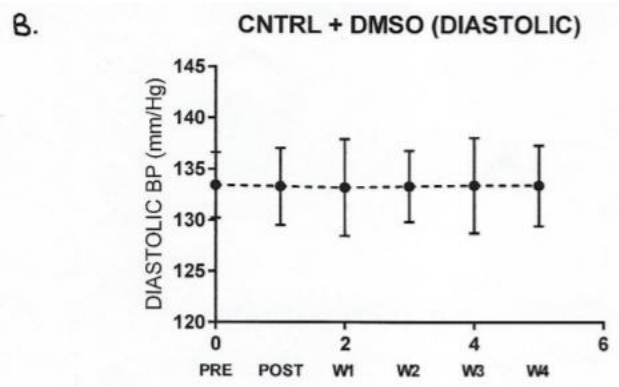
No changes in BP from pre-surgery to post-surgery in systolic, diastolic and MAP are expected. Also, no significant difference in BP from post-surgery (baseline) to week 4.

Figure 13. Predicted effects of DMSO in sham control group BP. A) systolic BP, B) diastolic BP and C) mean arterial blood pressure.

A.


CNTRL + DMSO (SYSTOLIC)
SYSTOLIC BP (mmHg)
190
180
170
160
150
0
2
4
6
PRE
POST
W1
W2
W3
W4

B.


CNTRL + DMSO (DIASTOLIC)
DIASTOLIC BP (mm/Hg)
145
140
135
130
125
120
0
2
4
6
PRE
POST
W1
W2
W3
W4

C.

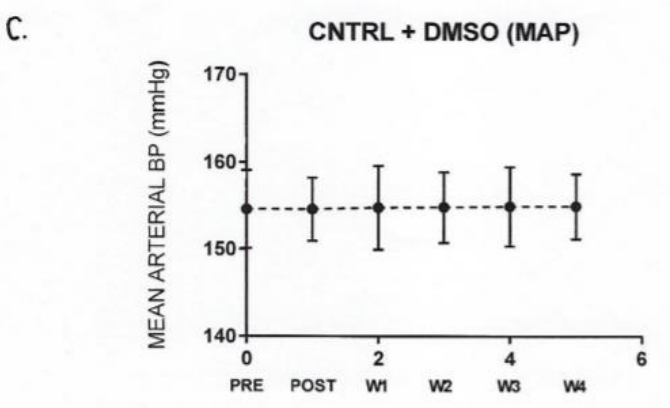

CNTRL + DMSO (MAP)
MEAN ARTERIAL BP (mmHg)
170
160
150
140
0
2
4
6
PRE
POST
W1
W2
W3
W4

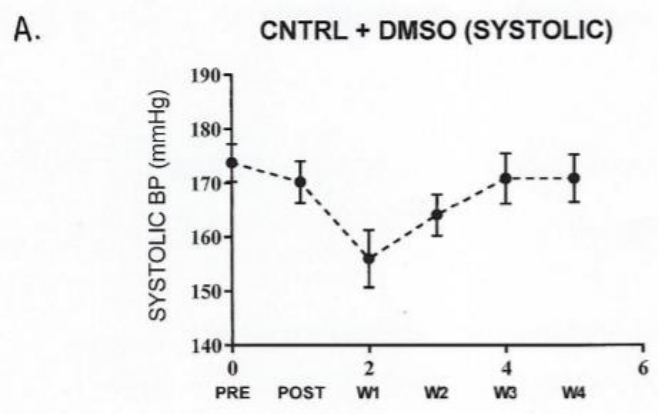
Figure 14 shows the experimental effects of DMSO in sham control group (n=4) on systolic, diastolic and mean arterial BP. Figure A shows systolic BP (mmHg) on Y axis and pre-surgery BP, post-surgery BP, W1 BP, W2 BP, W3 BP and W4 BP on X axis. Same axis is followed for figure B Diastolic BP and figure C- MAP. Error bars are standard error of the mean calculated as standard deviation/ $\sqrt{(n)}$.

Systolic BP has a $p>0.05$ hence is not significant across the experimental timeline. A significant decrease ($p \leq 0.05$) in BP from pre-surgery to post-surgery is seen in MAP, but not in systolic and diastolic BP. No significant difference ($p>0.05$) in BP from post-surgery to week 4 in systolic, diastolic and MAP is seen.

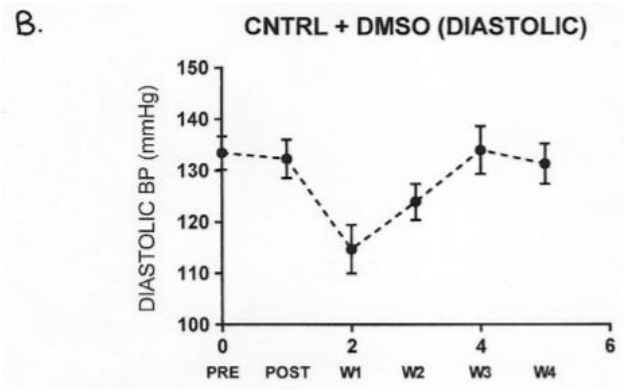
A drop in BP (systolic, diastolic and MAP) is seen at week 1. Systolic and diastolic BP recordings support our hypothesis as no final change in BP should be seen in control animals that were exposed to normoxia but MAP recordings oppose our hypothesis.

Figure 14. Experimental effects of DMSO in sham control group BP. A) systolic BP, B) diastolic BP and C) mean arterial blood pressure.

A.


CNTRL + DMSO (SYSTOLIC)
SYSTOLIC BP (mmHg)
190
180
170
160
150
140
0
2
4
6
PRE
POST
W1
W2
W3
W4

B.


CNTRL + DMSO (DIASTOLIC)
DIASTOLIC BP (mmHg)
150
140
130
120
110
100
0
2
4
6
PRE
POST
W1
W2
W3
W4

C.

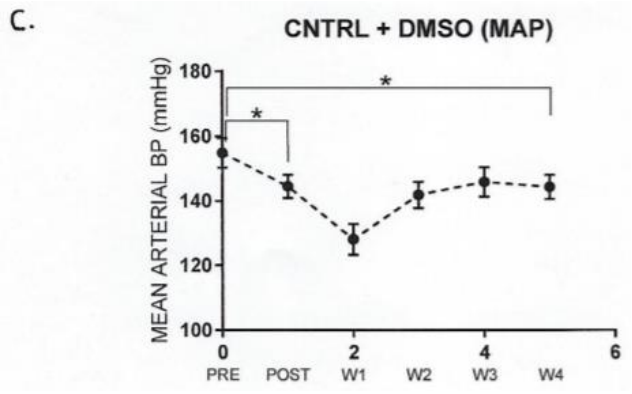
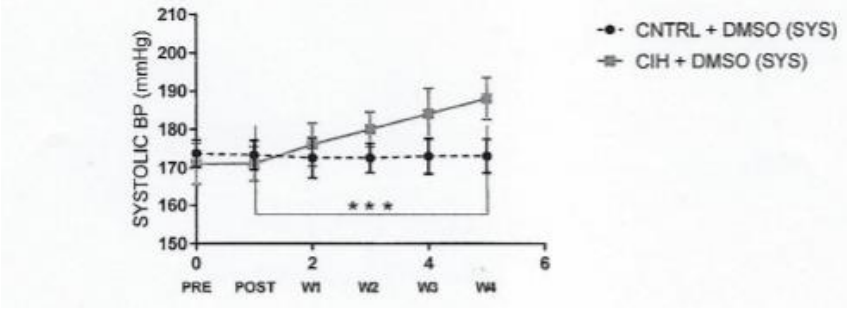

CNTRL + DMSO (MAP)
MEAN ARTERIAL BP (mmHg)
*
*
180
160
140
120
100
0
2
4
6
PRE
POST
W1
W2
W3
W4

Figure 15 shows Predicted effects of DMSO on systolic (n=8) (figure 15A), diastolic (n=8) (figure 15B) and MAP (n=8) (figure 15C) recordings of sham control group and CIH exposed group of animals. Figure A shows systolic BP (mmHg) on Y axis and pre-surgery BP, post-surgery BP, W1 BP, W2 BP, W3 BP and W4 BP on X axis. Same axis is followed for figure B Diastolic BP and figure C- MAP. Error bars are standard error of the mean calculated as standard deviation/ $\sqrt{(n)}$.

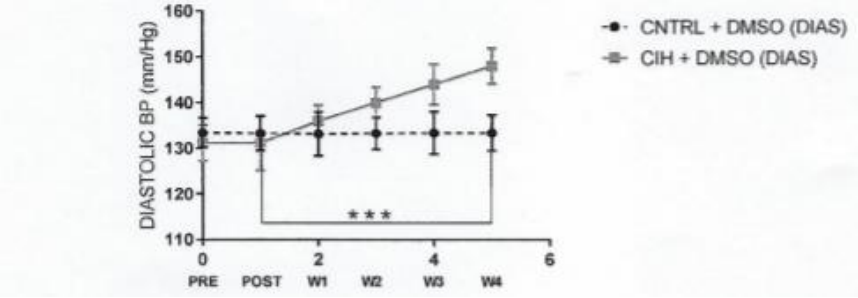
No significant difference is predicted from pre-surgery to post-surgery in systolic, diastolic and MAP for both sham control group and CIH exposed group. Also, no significant difference is predicted in systolic, diastolic and MAP from post-surgery (baseline) to week 4 for sham control group. A significant increase ($p \leq 0.001$) from post-surgery to week 4 is predicted in systolic, diastolic and MAP for CIH exposed group of animals in comparison to the controls.

Figure 15. Predicted effects of DMSO in sham control group and CIH exposed group of animals' BP. A) Systolic BP, B) Diastolic BP and C) MAP.

A. CNTRL+DMSO(SYSTOLIC) /CIH+DMSO (SYSTOLIC)



B. CNTRL + DMSO (DIASTOLIC)/CIH + DMSO (DIASTOLIC)



C. CNTRL + DMSO (MAP)/CIH + DMSO (MAP)

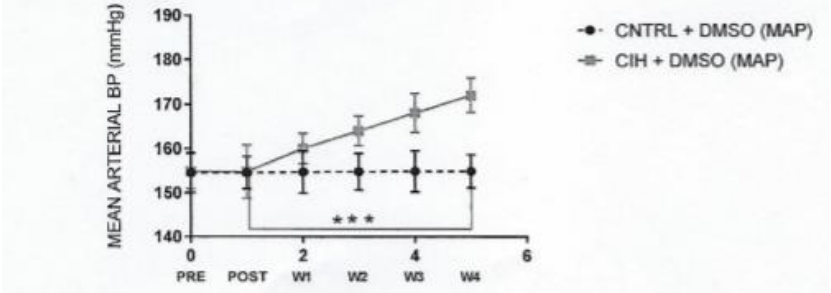


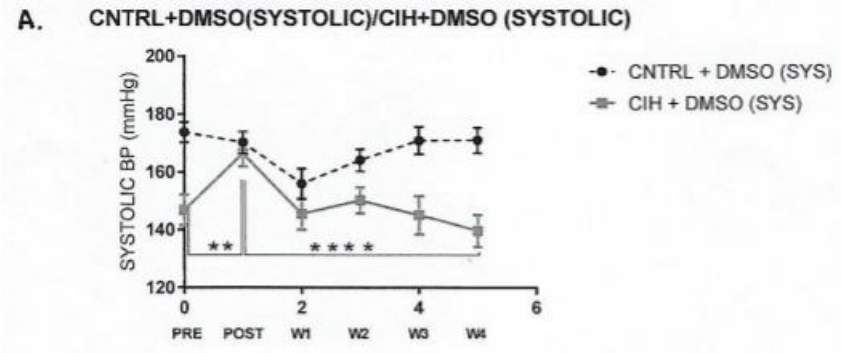
Figure 16 shows the experimental effects of DMSO in sham control group (n=4) and CIH exposed group of animals (n=4) on systolic, diastolic and MAP. Figure A shows systolic BP (mmHg) on Y axis and pre-surgery BP, post-surgery BP, W1 BP, W2 BP, W3 BP and W4 BP on X axis. Same axis is followed for figure B Diastolic BP and figure C- MAP. Error bars are standard error of the mean calculated as standard deviation/ $\sqrt{(n)}$.

No significant difference ($p>0.05$) is seen in systolic, diastolic and MAP for CIH exposed group over time. A significant decrease from post-surgery to week 4 in systolic, diastolic and MAP for CIH exposed group of animals is seen.

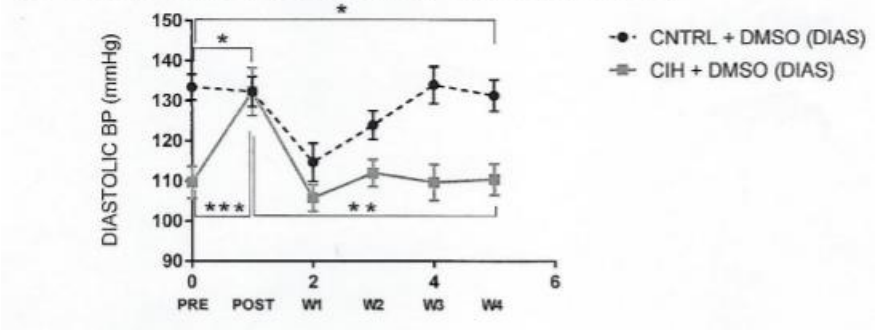
CIH + DMSO group opposes our hypothesis. They should have had an increase in BP in comparison to CNTRL + DMSO group as they were exposed to CIH and not treated with SST. CNTRL + DMSO group (systolic and diastolic) support our hypothesis.

Figure 16. Experimental effects of DMSO in sham control group and CIH exposed group of animals' BP. A) Systolic BP, B) Diastolic BP and C) MAP.

A. CNTRL+DMSO(SYSTOLIC)/CIH+DMSO (SYSTOLIC)



B. CNTRL + DMSO (DIASTOLIC)/CIH + DMSO (DIASTOLIC)



C. CNTRL + DMSO (MAP)/CIH + DMSO (MAP)

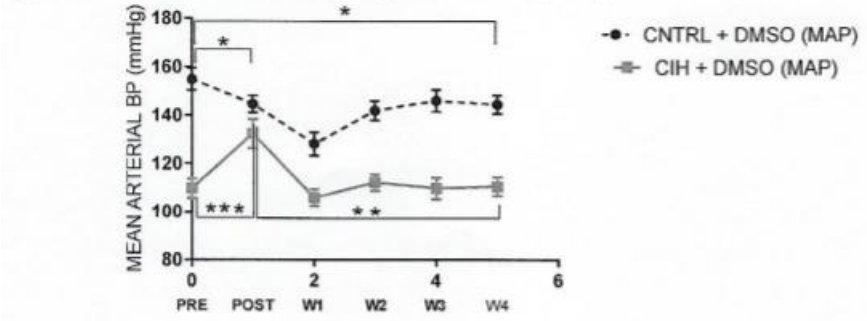
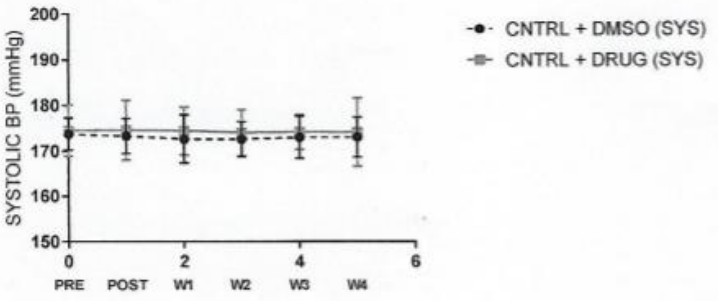


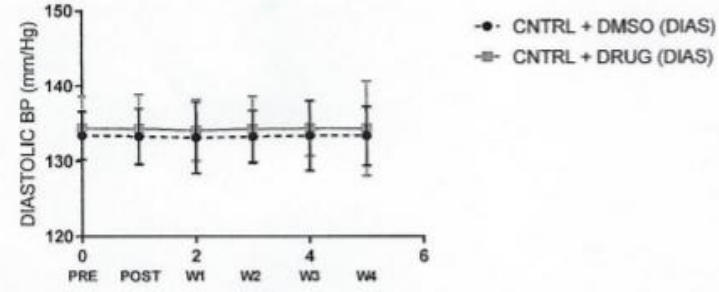
Figure 17 shows Predicted effects of DMSO (n=4) and drug (SST) (n=4) on systolic (n=8, $\bar{x} = 170.04\ vs\ 174.3$) (figure 17A), diastolic (n=8, $\bar{x}$ =133.33 vs 134.33) (figure 17B) and MAP (n=8, $\bar{x}$ =154.75 vs 154.75) (figure17C) in sham (normoxia) control group of animals. Figure A shows systolic BP (mmHg) on Y axis and pre-surgery BP, post-surgery BP, W1 BP, W2 BP, W3 BP and W4 BP on X axis. Same axis is followed for figure B Diastolic BP and figure C- MAP. Error bars are standard error of the mean calculated as standard deviation/ $\sqrt{(n)}$. No significant difference ($p>0.05$) from pre-surgery to post-surgery or from post-surgery (baseline) to week for systolic, diastolic and MAP for both DMSO and drug containing sham control group.

Figure 17. Predicted effects of DMSO and drug (SST) in sham control group of animals. A) Systolic BP, B) Diastolic BP and C) MAP.

A. CNTRL + DMSO (SYS)/CNTRL + DRUG (SYS)



B. CNTRL + DMSO (DIAS)/CNTRL + DRUG (DIAS)



C. CNTRL + DMSO (MAP)/CNTRL + DRUG (MAP)

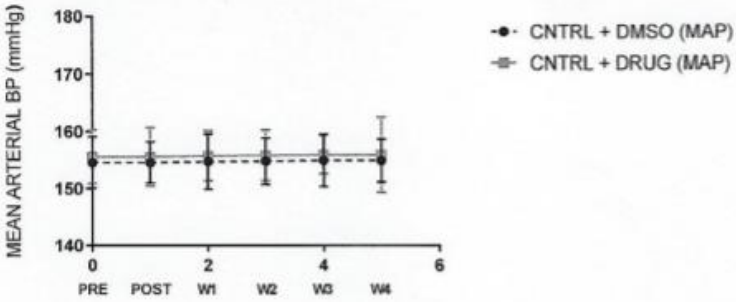
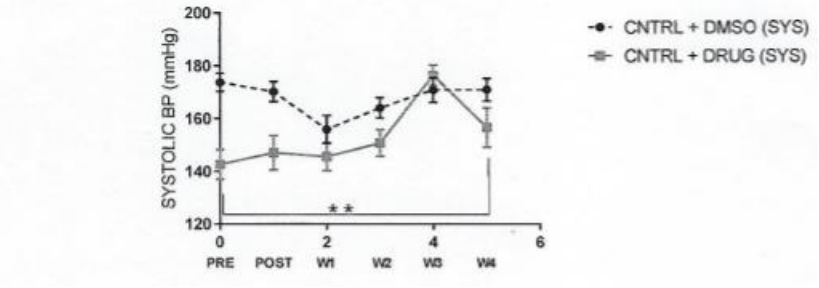


Figure 18 shows the experimental effects of DMSO (n=4) and drug (SST) (n=4) on systolic (n=8), diastolic (n=8) and mean arterial blood pressure (n=8). Figure A shows systolic BP (mmHg) on Y axis and pre-surgery BP, post-surgery BP, W1 BP, W2 BP, W3 BP and W4 BP on X axis. Same axis is followed for figure B Diastolic BP and figure C- MAP. Error bars are standard error of the mean calculated as standard deviation/ $\sqrt{(n)}$.

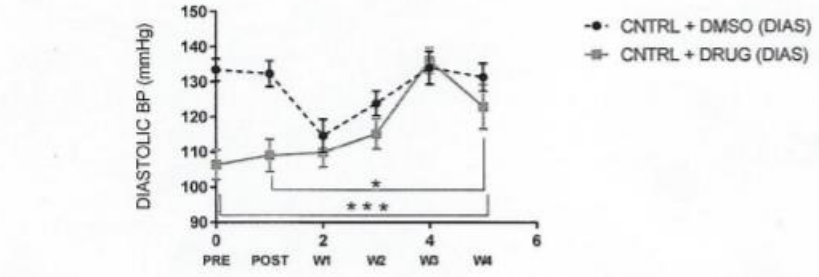
A significant increase ($p \leq 0.05$) is seen from pre-surgery to post-surgery in MAP for CNTRL + DMSO but none ($p > 0.05$) is seen from pre-surgery to post-surgery for systolic, diastolic and MAP for drug containing sham control group. No significant difference seen from pre-surgery baseline to week 4 in systolic, diastolic and MAP for DMSO containing sham control group. No significant difference seen from post-surgery to week 4 in systolic, but a significant difference ($p \leq 0.05$) seen in diastolic and MAP for drug containing sham control group. CNTRL + DRUG group opposes our hypothesis. They were expected to not show any change in BP as they were exposed to normoxia. Whereas, CNTRL + DMSO (systolic and diastolic BP) support our hypothesis.

Figure 18. Experimental effects of DMSO and drug (SST) in sham control group of animals. A) Systolic BP, B) Diastolic BP and C) MAP.

A. CNTRL + DMSO (SYSTOLIC)/CNTRL + DRUG (SYSTOLIC)



B. CNTRL + DMSO (DIASTOLIC)/CNTRL + DRUG (DIASTOLIC)



C. CNTRL + DMSO (MAP)/CNTRL + DRUG (MAP)

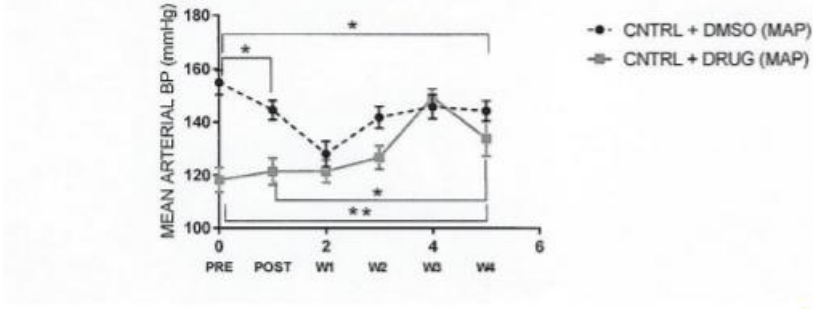
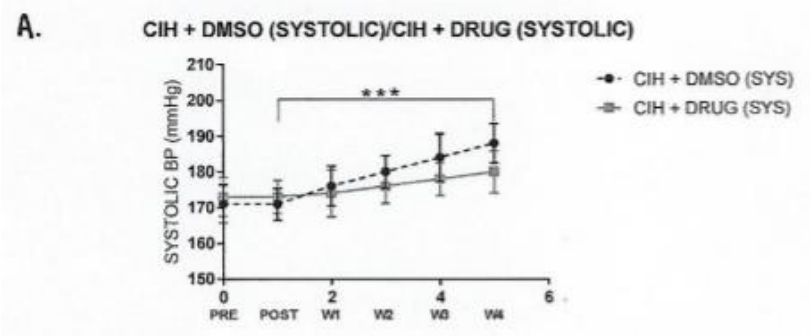


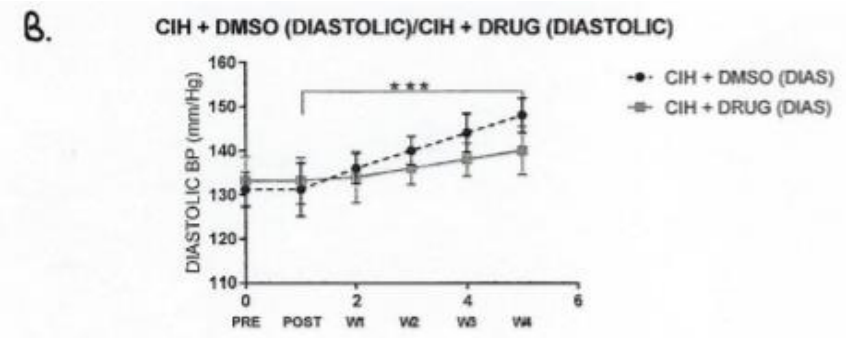
Figure 19 shows predicted effects of DMSO (n=4) and drug (SST) (n=4) on systolic (n=8, $\bar{x}$ =175.7 vs 178.36) (figure 17A), diastolic (n=8, $\bar{x}$ =135.73 vs 138.4) (figure 17B) and MAP (n=8, $\bar{x}$ =157.61 vs 162.28) (figure17C) in CIH exposed group of animals. Figure A shows systolic BP (mmHg) on Y axis and pre-surgery BP, post-surgery BP, W1 BP, W2 BP, W3 BP and W4 BP on X axis. Same axis is followed for figure B Diastolic BP and figure C- MAP. Error bars are standard error of the mean calculated as standard deviation/ $\sqrt{(n)}$. No significant difference ($p>0.05$) from pre-surgery to post-surgery in systolic, diastolic and MAP for both DMSO and drug containing CIH exposed groups. A significant increase ($p\leq0.001$) is predicted from post-surgery (baseline) to week 4 in systolic, diastolic and MAP for DMSO containing CIH exposed group. No significant difference ($p>0.05$) from post-surgery (baseline) to week 4 in systolic, diastolic and MAP for drug containing CIH exposed group of animals.

Figure 19. Predicted effects of DMSO and drug (SST) in CIH exposed group of animals. A) Systolic BP, B) Diastolic BP and C) MAP.

A.


CIH + DMSO (SYSTOLIC)/CIH + DRUG (SYSTOLIC)
SYSTOLIC BP (mmHg)
210
200
190
180
170
160
150
***
0
2
4
6
PRE
POST
W1
W2
W3
W4
CIH + DMSO (SYS)
CIH + DRUG (SYS)

B.


CIH + DMSO (DIASTOLIC)/CIH + DRUG (DIASTOLIC)
DIASTOLIC BP (mmHg)
160
150
140
130
120
110
***
0
2
4
6
PRE
POST
W1
W2
W3
W4
CIH + DMSO (DIAS)
CIH + DRUG (DIAS)

C.

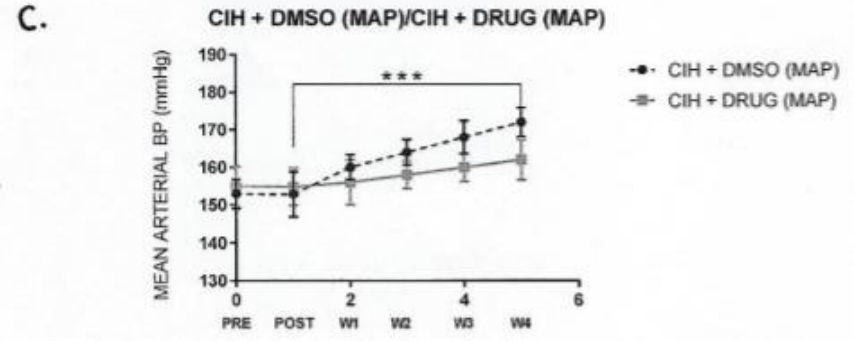

CIH + DMSO (MAP)/CIH + DRUG (MAP)
MEAN ARTERIAL BP (mmHg)
190
180
170
160
150
140
130
***
0
2
4
6
PRE
POST
W1
W2
W3
W4
CIH + DMSO (MAP)
CIH + DRUG (MAP)

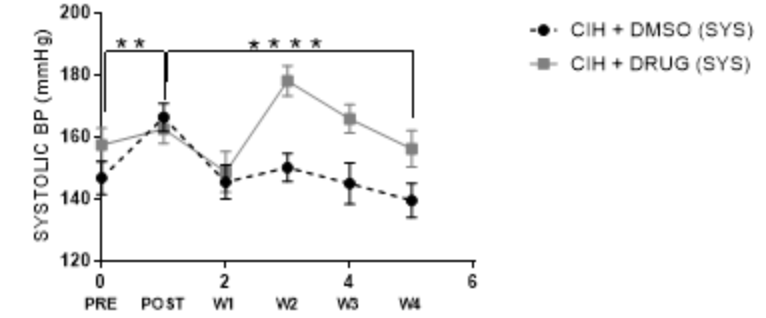
Figure 20 shows the experimental effects of DMSO (n=4) and drug (SST) (n=4) in CIH exposed group of animals, both treated (with SST) and not treated (with DMSO) on systolic, diastolic and mean arterial blood pressure. Figure A shows systolic BP (mmHg) on Y axis and pre-surgery BP, post-surgery BP, W1 BP, W2 BP, W3 BP and W4 BP on X axis. Same axis is followed for figure B Diastolic BP and figure C- MAP. Error bars are standard error of the mean calculated as standard deviation/ $\sqrt{(n)}$.

A significant increase from pre-surgery to post-surgery in systolic ($p \leq 0.01$), diastolic ($p \leq 0.001$) and MAP ($p \leq 0.001$) for DMSO containing CIH exposed group is seen. No significant difference ($p > 0.05$) is seen from pre-surgery in systolic, diastolic and MAP seen for drug containing CIH exposed group of animals. A significant decrease seen in systolic ($p \leq 0.0001$), diastolic ($p \leq 0.01$) and MAP ($p \leq 0.01$) from post-surgery (baseline) to week 4 in DMSO containing CIH exposed group but no significant difference ($p > 0.05$) seen in case of drug containing CIH exposed group of animals.

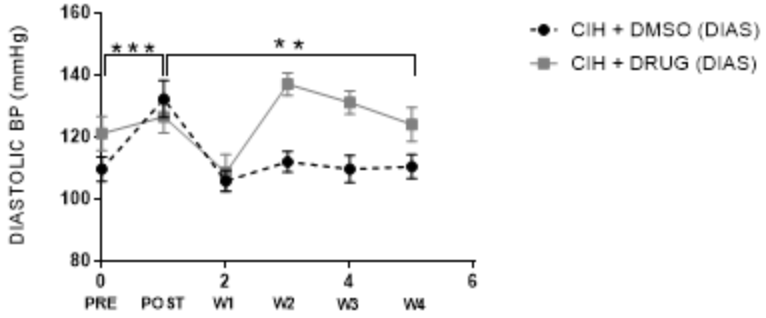
CIH + DMSO group of animals oppose our hypothesis. They should have had an increase in BP in comparison to CIH + SST group. CIH + SST also failed the hypothesis as it showed a significant increase in BP.

Figure 20. Experimental effects of DMSO and drug (SST) in CIH exposed group of animals. A) Systolic BP, B) Diastolic BP and C) MAP.

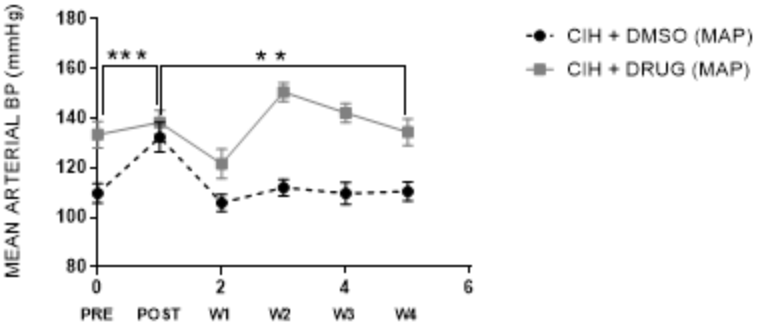
CIH + DMSO (SYSTOLIC)/CIH + DRUG (SYSTOLIC)



CIH + DMSO(DIASTOLIC)/CIH + DRUG (DIASTOLIC)



CIH + DMSO (MAP)/CIH + DRUG (MAP)



The only rats to show a significant increase from W1 to W2 were the CIH rats on the SST, which is the opposite of the desired effect. This group did show a descriptive decrease from W2 to W3 and from W3 to W4, although not a statistically significant decrease. The control rats on the SST showed significant increases from Pre to W3, Post to W3, W1 to W3, and W2 to W3.

DISCUSSION

Sleep apnea is a clinically prevalent disorder in which breathing repeatedly stops and starts every 15-30 seconds (Nieto FJ et al., 2000; Young T et al., 1993.; Ramirez JM et al., 2013).

The obstruction of the upper airway during sleep causes termination of air flow leading to intermittent hypoxia and affects an estimated 10 % of the population in the US (Nanduri et al., 2017). Patients suffering from SA showcase emanations involving hypertension, myocardial ischemia, stroke and even unanticipated death (Sica et al., 2000). Patients with OSA get mainly treated with CPAP which is known to improve oxygenation (Peng et al., 2017) but is not significantly effective in some patients, while some find it difficult to tolerate (Peng et al., 2017). An exaggerated CB chemosensory reflex in humans and rodents has been shown to contribute to IH-induced hypertension (Prabhakar et al., 2015).

When exposed to IH, rodents have shown to mimic the blood O_2 saturation profiles during OSA and exhibit hypertension (Fletcher et al., 1995; Kumar et al., 2006; Peng et al., 2006; Troncoso Brindeiro et al., 2007). The level of O_2 desaturation is parallel to elevation of systolic BP during SA (Shepard et al., 1985).

Based on all these findings and the findings by Dr. Wilson, we hypothesized that SST analogues given *in-vitro* will attenuate the response of carotid body to intermittent hypoxia and then prevent carotid body sensitization, thus preventing systemic hypertension from developing during intermittent hypoxia.

Important Findings

CONTROL + DMSO: Since the animals were not exposed to CIH, they should not be hypertensive. Also, as they were not given any treatment drug (SST), we should see no change in BP from W1 to W4. Although, we might expect to see some changes in BP post-surgery.

The observed drop in BP could be due to the use of anesthesia, hypovolemic shock, septic shock. A rise in BP can be a result of pain or stress. As per the actual data collected, interestingly, we see a significant drop in BP at W1 in all animals. In all animals that had surgery, all treated (Octreotide) and control groups, show a significant hypotension following surgery. We also see a significant drop in MAP in CONTROL + DMSO group of rats from Pre to Post (which could be due to septic shock) and an overall drop in BP from Pre-surgery to W4. The sudden drop in BP at W1 could have possibly not allowed the W4 BP to rise back to its normal BP levels. But we see no significant change in BP from post-surgery to W4 in Systolic, Diastolic and MAP. Fletcher et al., 1992 performed a similar experiment with almost same protocol and showed that the animals become hypertensive. BP was indirectly measured in unconscious rats using an inflatable tail-cuff and photoelectric sensor. It was different from our experiment as they did not perform any surgeries or implant minipumps.

CIH + DMSO: Animals should become hypertensive which means their BP should increase. Also, they were given the vehicle- DMSO, instead of the drug, SST, therefore we expected to see an increase in BP. Prabhakar et al., 2017 provided evidence that unlike ST (short term)-IH (10 days exposure to CIH), LT (long term)-IH (30 days) leads to

irreversible changes in elevated BP and irregular breathing and these are associated with persistent activation of the CB chemosensory reflex.

For this group of animals, we saw a significant increase in BP from pre-surgery to post-surgery which I speculate can be a result of pain or distress in animals due to surgery. A constant drop in BP at W1 is seen in this group of rats as well. We also see a drop in BP from post-surgery to W4 which is exact opposite of what we expected. Surprisingly, from pre-surgery to W4, there is no significant difference in systolic, diastolic or MAP. One could think that the CIH system may have been ineffective but that cannot be true. The effectiveness of the system was ensured when we performed CIH controls with no surgery before proceeding with the actual experiment. Figure 18 shows experimental results (n=6) for *in-vivo* studies of animals that did not undergo surgery. Here, we see a significant increase in systolic, diastolic and MAP of CIH control rats from W3 to W4.

CONTROL + DRUG: This is the group of rats that were surgically implanted with pumps containing SST analogue but were not exposed to CIH. We expected them to not get hypertensive, therefore no increase in BP and therefore the drug should not work. They should behave like the controls with no drug. While analyzing the actual data, we saw that the drug had effects opposite to what we expected. There was a significant rise in BP from post-surgery to W4. It should have been the opposite (Figure 15). MAP shows a significant change in pre and post-surgery baseline and no significant change from post-surgery to W4 BP measurements.

Final average group B [CNTRL + SST] (in gray) shows no significant change in BP from post-surgery to W4. For diastolic and MAP, there is a significant change in BP from post-surgery to W4.

As the time frame of the experiment was limited by the lifespan of the pump. It is also strange that the control rats on SST showed significant increases from Pre to W3, Post to W3, W1 to W3 and W2 to W3. This group would have the least likely to show any increase in SBP of the three groups if the SST is effective. (see appendix).

CIH + DRUG: We expect the animals to become hypertensive as they were exposed to CIH, which is why their BPs should increase since they were treated with drug-SST. Hoffman et al., 1989 demonstrated an increase in BP in animals with osmotic pumps containing SST analogues (dissolved in 0.9% saline) at a rate of 10µg/kg/h for 7 days in comparison to the controls with 0.9% NaCl in their pumps.

Systolic BP for adult male Sprague Dawley rats should be in the range of 120-130mmHg but our rats' systolic BP was in the range of 160-170mmHg which means our rats were already hypertensive. They could have been hypertensive because of our approach to BP measurement. Non-invasive tail-cuff plethysmography is relatively less expensive and does not require any surgical implantations, but it also does not give precise results. Investigators have identified the technical factors that most likely affect the accuracy of tail-cuff measurements while simultaneously recording BP using telemetry. These include entry of the investigator into the room where the measurements were performed, moving the cages around, handling the rodent, restraint of the rodent and inflation of the cuff on the tail. Changes in the BP and heart rate induced by restraint stress and tail-cuff inflation did not diminish in magnitude, even after many repeated trials. Telemetry on the other hand is the "gold standard" of BP measurement (Gregory D. Fink, Editorial, AHA, ASA).

Future experiments

The unexpected drop in BP post-surgery may have prevented the rise of BP in CIH + SST animals and to avoid any discrepancies in the results, any surgical implantations prior to BP recordings should be avoided and alternative ways of drug delivery should be considered. Use of depot injections to avoid unnecessary fluctuations in BP could be used. Also, the use of CODA non-invasive tail cuff BP measurement system was not the best choice. Fletcher and his colleagues observed an increase in MAP in rodents exposed to CIH for 7 h/day for 35 days during their diurnal sleep (Fletcher et al., 1992b)). They placed indwelling arterial catheter to measure BP. Next time, a greater number of animals and the use of telemetry for BP recordings can give us better and more accurate results.

Potential Pitfalls

In many studies, Octreotide has been used to treat acromegaly and esophageal variceal hemorrhage in humans mainly as bolus injections of 50µg followed by 25-50µg/h (Reynaert et al., 2003). Some also use Long Acting Release (LAR) Octreotide by deep subcutaneous depot injections to administer a dose of 20mg-40mg/month (Colao et al., 2009; Tolis et al., 2006). However, to study Long-Term (LT) effects of SST or SSTA, a dose of 10µg/kg/h or 8mg/kg/month is considered a standard protocol (Schmid et al., 2012). In the current project, we intended to use 10µg/kg/h of SSTA concentration but due to wrong calculations we ended up using 8.5µg/kg/h.

With a smaller size in comparison to humans, the basal metabolic rate- the rate of energy production over a fixed period of time under constant conditions- is much less than that of man, because of less body mass. However, the mass-specific rate, that is, the basal

metabolic rate per gram of body weight is known to be seven times greater in rodents than in humans (Lloyd et al., 2005). As a consequence, it is likely that rats will metabolize Octreotide drug faster than humans.

Increasing the dose of Octreotide should help mitigate the problems caused by high metabolic rate and increased rate of drug metabolism. Three conventional concentrations of drug- 10µg/Kg/h, 30µg/Kg/h and 100µg/Kg/h could be used.

The subtype of SSTRs on the carotid body and carotid sinus nerve are not known but we do know that the SST analogue Octreotide has high affinity for SSTR2 and SSTR5 and some affinity for SSTR3 (See Table 1). There is a possibility that the CB/CSN may not have any SSTR 2, 3 and 5 which means Octreotide would never work. To avoid this, other SST analogues such as Vapreotide or Somatuline could be tested. They have affinities for SSTR 2 through 5.

APPENDIX

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DATE: 13 February 2018

TO: Kamra, Kajal

FROM: Mike Bottomley

SUBJECT: Final Report for CIH Analysis

Dear Kajal,

Here are the results of the analysis for your chronic intermittent hypoxia (CIH) data. You are interested in determining if a drug (SST) has any effect on various blood pressure measures of rats undergoing CIH. You have four treatment groups:

(i) Normal oxygen conditions and placebo

(ii) Normal oxygen conditions and SST

(iii) CIH and placebo

(iv) CIH and SST

To simulate CIH the rats were placed in a chamber that was intermittently flooded with nitrogen to lower the oxygen content, and shortly thereafter, the oxygen content was returned to a regular level. Rats on the drug and rats not on the drug were present in all

cages. The cages that were not exposed to the CIH conditions had a device that mimics the sound of the nitrogen blast in order to keep the conditions as similar as possible.

You recorded the systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP) of the rats. The rats were measured at the start of the experiment, after a pump was surgically implanted, and then once a week for four weeks. Five measurements were taken at each time point. Due to complications arising from the surgery that implanted the pumps, some of the rats had to be removed from the experiment before the end of the study. Rats that were removed within a week after the surgery were excluded from the analysis since the drug would not have had ample time to show an effect.

For each of the three response variables a repeated measures mixed effects ANOVA with a random effect for rat ID was run. The repeated measures aspect takes the correlation between time points for each rat into effect and the random effect factors in the correlation for the 5 measurements on each rat at each of the time points. A level of significance of alpha = 0.05 was used for each model. Post hoc pairwise comparisons were made via Tukey's multiple comparison procedure, which controls for an inflated type I error rate (rejecting the null hypothesis of no difference when in reality there actually is no difference) that can arise when multiple comparisons are made. This procedure guarantees that the experiment-wise type I error rate for each of the three models is at most alpha = 0.05. All analyses were performed on SAS version 9.4. Descriptive statistics for each of the variables at each time point for each of the treatment groups are given below in Table 1.

Group	Time	No. of Obs	Variable	Label	N	Mean	Std Dev	Minimum	Maximum	N Miss
CNTRL + DMSO	Pre	35	SBP DBP MAP	SBP DBP MAP	35 35 35	169.11 129.11 149.29	23.79 22.28 28.26	107.00 80.00 89.00	210.00 175.00 210.00	0 0 0
	Post	35	SBP DBP MAP	SBP DBP MAP	35 35 35	170.89 132.60 144.94	21.38 19.86 19.02	134.00 108.00 120.00	217.00 180.00 189.00	0 0 0
	W1	35	SBP DBP MAP	SBP DBP MAP	35 35 35	148.11 110.17 122.46	33.21 26.66 28.16	95.00 73.00 84.00	203.00 163.00 176.00	0 0 0
	W2	35	SBP DBP MAP	SBP DBP MAP	35 35 35	161.49 121.23 138.46	20.55 19.49 22.59	135.00 91.00 108.00	214.00 177.00 187.00	0 0 0
	W3	35	SBP DBP MAP	SBP DBP MAP	30 30 30	170.83 134.03 145.87	25.40 25.56 25.07	131.00 90.00 105.00	218.00 192.00 200.00	5 5 5
	W4	35	SBP DBP MAP	SBP DBP MAP	30 30 30	170.87 131.37 144.27	24.07 21.55 20.64	117.00 74.00 96.00	218.00 174.00 188.00	5 5 5
CIH + DMSO	Pre	35	SBP DBP MAP	SBP DBP MAP	35 35 35	151.49 113.43 125.77	28.81 21.03 23.18	96.00 79.00 84.00	201.00 154.00 166.00	0 0 0

Group	Time	No. of Obs	Variable	Label	N	Mean	Std Dev	Minimum	Maximum	N Miss
	Post	35	SBP DBP MAP	SBP DBP MAP	35 35 35	156.09 119.49 131.37	27.40 34.24 30.93	106.00 56.00 73.00	204.00 194.00 197.00	0 0 0
	W1	35	SBP DBP MAP	SBP DBP MAP	35 35 35	141.03 105.63 117.03	37.53 24.33 28.17	70.00 53.00 58.00	191.00 149.00 163.00	0 0 0
	W2	35	SBP DBP MAP	SBP DBP MAP	35 35 35	160.89 119.37 132.83	27.34 19.34 21.27	114.00 86.00 96.00	218.00 154.00 164.00	0 0 0
	W3	35	SBP DBP MAP	SBP DBP MAP	35 35 35	156.31 119.06 131.17	33.68 24.52 27.05	99.00 75.00 85.00	231.00 161.00 184.00	0 0 0
	W4	35	SBP DBP MAP	SBP DBP MAP	25 25 25	139.64 110.48 119.92	27.61 19.40 21.51	84.00 74.00 77.00	178.00 140.00 152.00	10 10 10
CNTRL + DRUG	Pre	35	SBP DBP MAP	SBP DBP MAP	35 35 35	142.63 106.40 118.17	33.25 25.13 27.63	94.00 69.00 79.00	197.00 147.00 160.00	0 0 0
	Post	35	SBP DBP MAP	SBP DBP MAP	35 35 35	147.06 109.09 121.34	38.69 27.20 30.25	37.00 32.00 33.00	211.00 170.00 183.00	0 0 0

Group	Time	No. of Obs	Variable	Label	N	Mean	Std Dev	Minimum	Maximum	N Miss
	W1	35	SBP DBP MAP	SBP DBP MAP	35 35 35	145.63 109.94 121.46	31.04 24.35 25.85	87.00 66.00 74.00	203.00 180.00 187.00	0 0 0
	W2	35	SBP DBP MAP	SBP DBP MAP	35 35 35	150.80 115.23 126.69	29.80 25.89 26.27	89.00 53.00 79.00	194.00 166.00 174.00	0 0 0
	W3	35	SBP DBP MAP	SBP DBP MAP	35 35 35	176.34 136.00 149.20	22.69 21.77 19.88	137.00 100.00 117.00	222.00 195.00 202.00	0 0 0
	W4	35	SBP DBP MAP	SBP DBP MAP	35 35 35	156.66 122.94 133.83	43.95 37.20 38.96	82.00 61.00 68.00	230.00 181.00 197.00	0 0 0
CIH + DRUG	Pre	25	SBP DBP MAP	SBP DBP MAP	25 25 25	157.60 121.16 133.40	27.04 27.57 26.94	106.00 67.00 80.00	194.00 162.00 170.00	0 0 0
	Post	25	SBP DBP MAP	SBP DBP MAP	25 25 25	162.68 126.60 138.32	23.31 26.43 24.76	116.00 89.00 98.00	204.00 194.00 197.00	0 0 0
	W1	25	SBP DBP MAP	SBP DBP MAP	25 25 25	148.80 108.60 121.64	32.83 29.36 29.75	104.00 78.00 86.00	237.00 189.00 197.00	0 0 0

Group	Time	No. of Obs	Variable	Label	N	Mean	Std Dev	Minimum	Maximum	N Miss
	W2	25	SBP	SBP	25	178.24	24.48	136.00	236.00	0
			DBP	DBP	25	18.41	112.00	188.00	0	
			MAP	MAP	25	137.12	18.71	120.00	193.00	0
					25	150.56				
	W3	25	SBP	SBP	25	165.96	23.15	129.00	217.00	0
			DBP	DBP	25	18.63	100.00	174.00	0	
			MAP	MAP	25	131.16	19.00	109.00	183.00	0
					25	142.12				
	W4	25	SBP	SBP	25	156.28	29.38	90.00	204.00	0
			DBP	DBP	25	27.29	57.00	169.00	0	
			MAP	MAP	25	124.08	26.88	69.00	178.00	0
					25	134.40				

Table 1: Descriptive statistics for CIH SST data by treatment group and time

The following three sections detail the results for each of the three response variables. In each of the three models, presence of a significant interaction between treatment group and time was detected ($P < 0.0001$ for each model). This is evidence that any potential treatment effects are not constant across time. As a result, the time points had to be compared individually. This resulted in 276 pairwise comparisons being made for each of the three models that were run. Using a level of alpha = 0.05, with 276 comparisons you would expect to commit around 14 type I errors by chance, which is why I chose to use Tukey's multiple comparison procedure. The interaction is evidenced in the interaction plots (Figures 1-3) by the non-parallel lines. If the treatment effect were constant across

all time points the lines would be approximately parallel and the interaction term would not be statistically significant.

I. Systolic Blood Pressure

Of the 276 pairwise comparisons, those that had significant adjusted *P*-values are given below in Table 2. Positive estimated mean differences indicate the first value was higher, negative estimated mean differences indicate the second value was higher. The adjusted 95% confidence intervals give a range of plausible values for the true mean difference. These intervals appear somewhat wide. This is likely due to the adjustment made to correct for the multiple comparisons, as well as having a relatively small sample size that displayed a high amount of variance. The order of subtraction is the same as the order of listing in the "Comparison" column. The interaction plot follows in Figure 1.

Comparison	Estimated Mean Difference	Adjusted 95% Confidence Interval	Adjusted *P*-value
CIH + DRUG W1 - CIH + DRUG W2	-29.44	(-56.99, -1.89)	0.0211
CNTRL + DMSO Post - CNTRL + DMSO W1	22.77	(1.75, 43.80)	0.0174
CNTRL + DRUG Post - CNTRL + DRUG W3	-29.29	(-50.66, -7.91)	0.0002
CNTRL + DRUG Pre - CNTRL + DRUG W3	-33.71	(-55.15, -12.28)	< 0.0001
CNTRL + DRUG W1 - CNTRL + DRUG W3	-30.71	(-51.74, -9.69)	< 0.0001
CNTRL + DRUG W2 - CNTRL + DRUG W3	-25.54	(-48.83, -2.26)	0.0144

Table 2: All significant pairwise comparisons for SBP

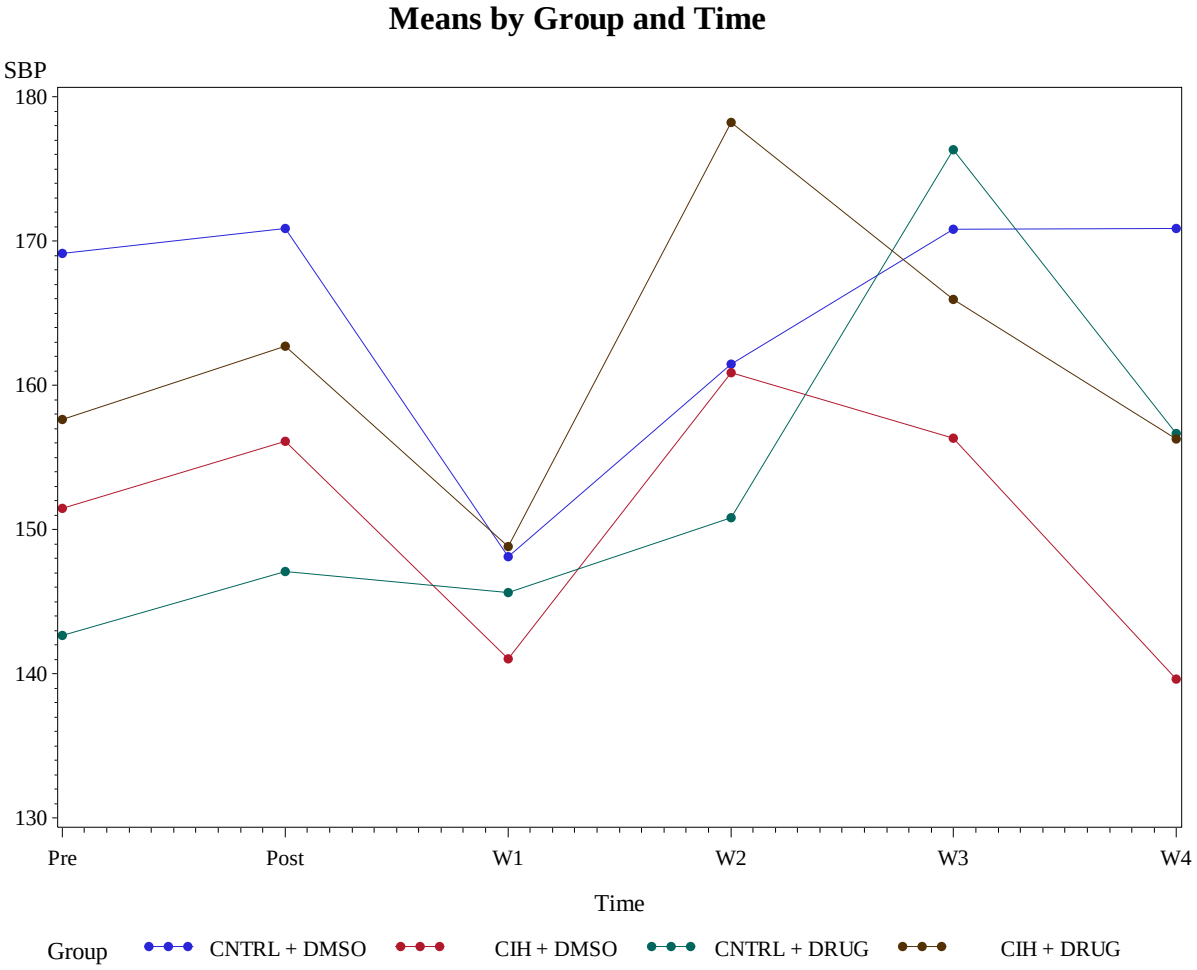

Figure 1: Interaction plot for SBP

As we discussed at one of our meetings, some of these results are rather curious. Descriptively, all of the rats displayed a decrease from Post to W1 (whether or not it was a statistically significant decrease), which is to be expected while they recover from the surgery. However, the only rats to show a significant increase from W1 to W2 were the CIH rats on the SST, which is the opposite of the desired effect. However, it could be possible that the SST takes time to start working (based on your decision to exclude rats who were removed before W2. This group did show a descriptive decrease from W2 to

W3 and from W3 to W4, although not a statistically significant decrease. Perhaps with a larger sample size and fewer pairwise comparisons this would become statistically significant. It is also possible that the drug did not have ample time to take full effect and given more time, this downward trend might have continued (note that this is pure speculation). I think you mentioned the timeframe of the experiment was limited by the lifespan of the pump. It is also strange that the control rats on the SST showed significant increases from Pre to W3, Post to W3, W1 to W3, and W2 to W3. It would seem that this group would have been the least likely to show any increase in SBP of the three groups if the SST is effective.

II. Diastolic Blood Pressure

Comparison	Estimated Mean Difference	Adjusted 95% Confidence Interval	Adjusted *P*-value
CIH + DRUG W1 - CIH + DRUG W2	-28.52	(-50.80, -6.24)	0.0010
CIH + DRUG W1 - CIH + DRUG W3	-22.56	(-44.32, -0.80)	0.0321
CNTRL + DMSO Post - CNTRL + DMSO W1	22.43	(1.22, 43.64)	0.0247
CNTRL + DMSO W1 - CNTRL + DMSO W3	-20.71	(-39.97, -1.45)	0.0196
CNTRL + DRUG Post - CNTRL + DRUG W3	-26.91	(-48.88, -4.95)	0.0023
CNTRL + DRUG Pre - CNTRL + DRUG W3	-29.60	(-47.63, -11.57)	< 0.001
CNTRL + DRUG W1 - CNTRL + DRUG W3	-26.06	(-44.45, -7.66)	<0.0001
CNTRL + DRUG W2 - CNTRL + DRUG W3	-20.77	(-37.05, -4.49)	0.0010

Table 3: All significant pairwise comparisons for DBP

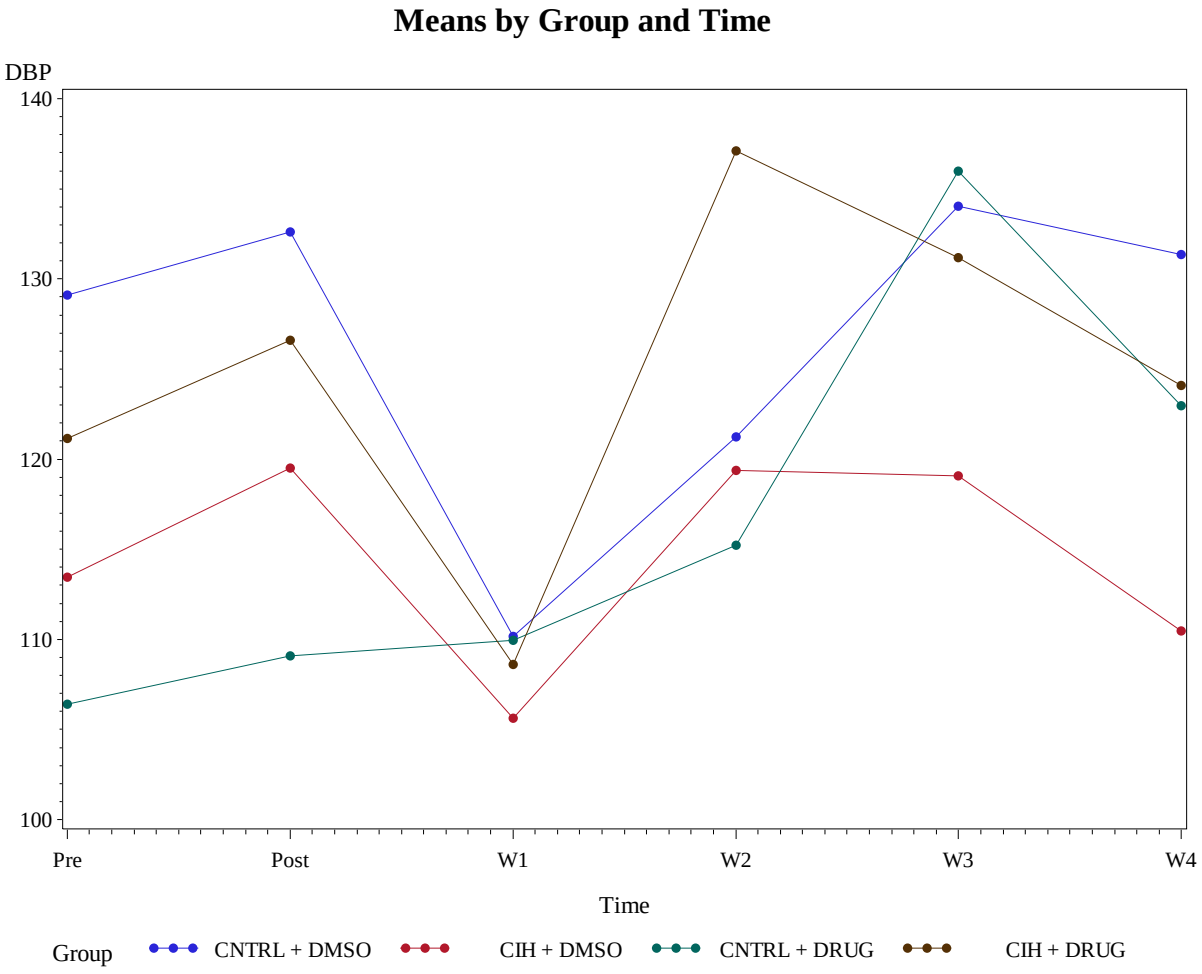

Figure 2: Interaction plot for DBP

All significant pairwise comparisons for SBP were also significant for DBP. Two additional pairwise comparisons were also significant. CIH rats on SST had a significantly higher mean DBP at W3 compared to W1. Control oxygen rats on the placebo had a significantly higher mean DBP at W3 compared to W1.

III. Mean Arterial Pressure

Comparison	Estimated Mean Difference	Adjusted 95% Confidence Interval	Adjusted *P*-value
CIH + DRUG W1 - CIH + DRUG W2	-28.92	(-52.18, -5.66)	0.0017
CNTRL + DMSO Post - CNTRL + DMSO W1	22.49	(1.20, 43.77)	0.0250
CNTRL + DMSO Pre - CNTRL + DMSO W1	26.83	(5.72, 47.94)	0.0011
CNTRL + DRUG Post - CNTRL + DRUG W3	-27.86	(-50.43, -5.29)	0.0020
CNTRL + DRUG Pre - CNTRL + DRUG W3	-31.03	(-50.96, -11.10)	< 0.0001
CNTRL + DRUG W1 - CNTRL + DRUG W3	-27.74	(-46.35, -9.14)	< 0.0001
CNTRL + DRUG W2 - CNTRL + DRUG W3	-22.51	(-39.00, -6.03)	0.0002

Table 4: All significant pairwise comparisons for MAP

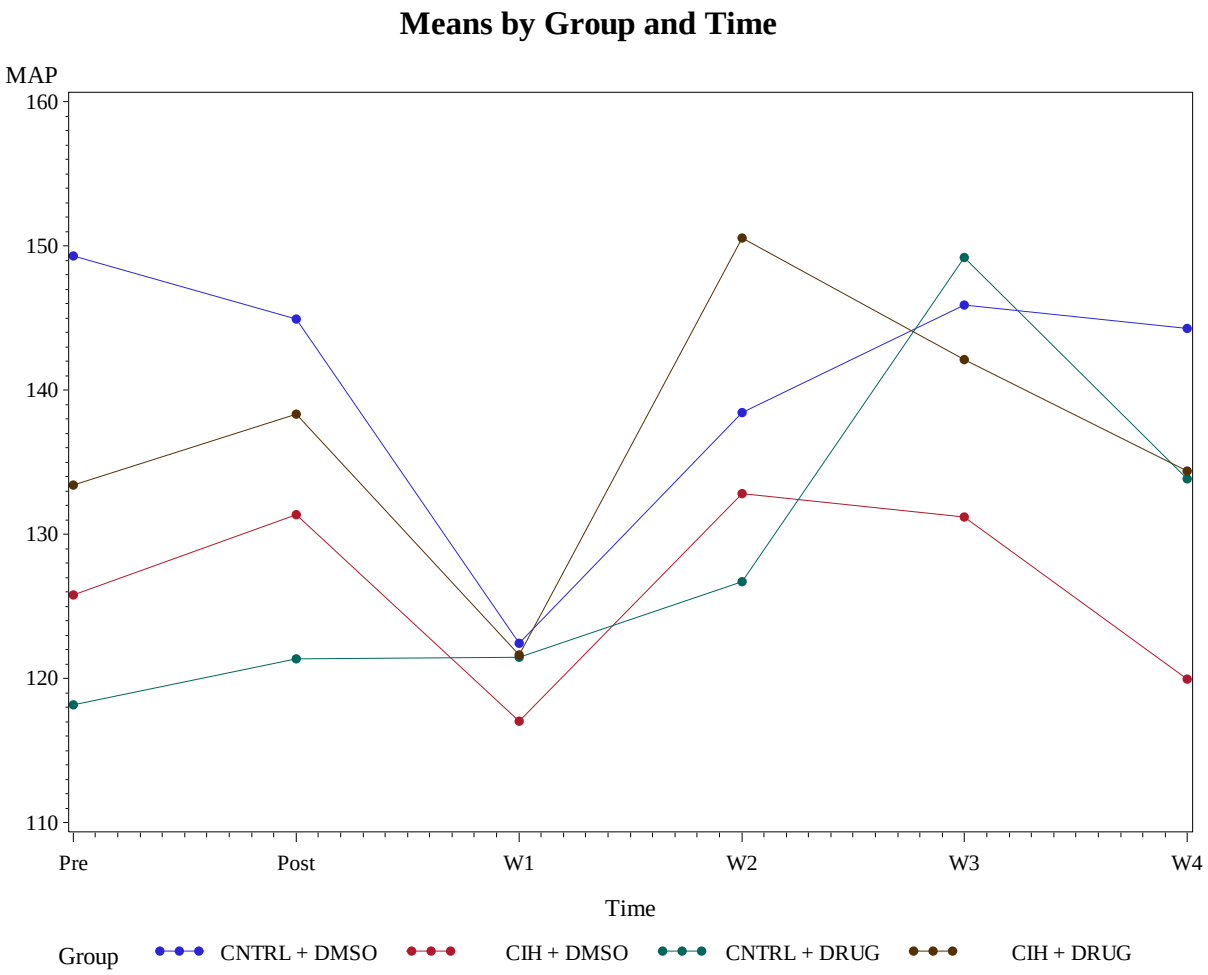

Figure 3: Interaction plot for MAP

MAP shared many of the same pairwise comparisons as SBP and DBP. One that was different was the mean MAP was significantly higher Pre compared to W1 for the control oxygen plus placebo group.

Let me know if you have any questions about anything contained in this report.

Sincerely,

Mike Bottomley

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