CREATING A SEPTIC SHOCK MODEL IN ADULT WISTAR RATS AND OBSERVING THE EFFECT OF NORADRENALINE

A dissertation submitted in partial fulfilment of the requirements for the degree of Doctor of Medicine in Physiology (Branch V) of the Tamil Nadu Dr. M. G. R. Medical University, Chennai – 600 032

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CERTIFICATE

This is to certify that this dissertation work titled **Creating A Septic Shock Model in Adult Wistar Rats And Observing The Effect Of Noradrenaline** is a bona fide, original work of the candidate **Dr. Sajo Thomas** with registration number **201715353**, for the partial fulfillment of the rules and regulations for the **Doctor of Medicine (M.D.)** in the branch of **Physiology (Branch V)** examination to be held in May 2020 by the Tamil Nadu Dr. MGR Medical University, Chennai. This thesis has not been submitted, in part or full, to any other university.

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DECLARATION

I hereby declare that the investigations that form the subject matter of this dissertation titled **Creating A Septic Shock Model In Adult Wistar Rats And Observing The Effect Of Noradrenaline**, was carried out by me during my term as a post graduate student in the **Department of Physiology**, Christian Medical College, Vellore. This thesis has not been submitted in part or full to any other university.

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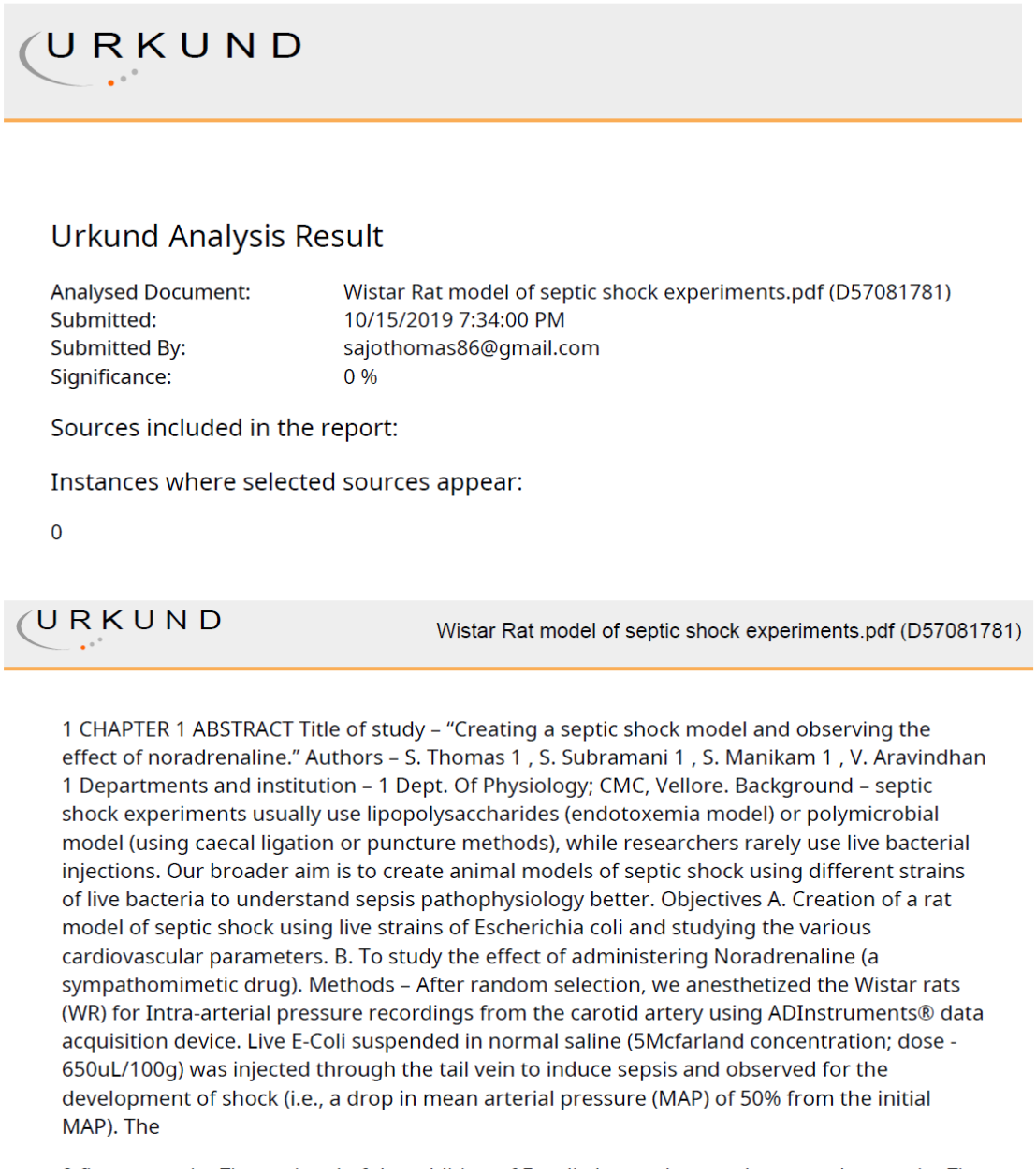

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1 CHAPTER 1 ABSTRACT Title of study – "Creating a septic shock model and observing the effect of noradrenaline." Authors – S. Thomas 1 , S. Subramani 1 , S. Manikam 1 , V. Aravindhan 1 Departments and institution – 1 Dept. Of Physiology; CMC, Vellore. Background – septic shock experiments usually use lipopolysaccharides (endotoxemia model) or polymicrobial model (using caecal ligation or puncture methods), while researchers rarely use live bacterial injections. Our broader aim is to create animal models of septic shock using different strains of live bacteria to understand sepsis pathophysiology better. Objectives A. Creation of a rat model of septic shock using live strains of Escherichia coli and studying the various cardiovascular parameters. B. To study the effect of administering Noradrenaline (a sympathomimetic drug). Methods – After random selection, we anesthetized the Wistar rats (WR) for Intra-arterial pressure recordings from the carotid artery using ADInstruments® data acquisition device. Live E-Coli suspended in normal saline (5Mcfarland concentration; dose - 650uL/100g) was injected through the tail vein to induce sepsis and observed for the development of shock (i.e., a drop in mean arterial pressure (MAP) of 50% from the initial MAP). The

ACKNOWLEDGMENT

Research! And what are we searching? That is one question I had regarding research. I used to think, all essential aspects of science are already established beyond any shadow of a doubt, and that there is an elite class of scientists who do research. That is why we have textbooks, you see!

Moreover, I thought, our job as students is to attend class, read the given text, and spew out answers on a test. Only later did I realize that we live in a day and age where a standard medical textbook is almost outdated in a few years. That is simply because students of science keep pushing the limits of knowing relentlessly. I have found some people in my life who have opened a new world of Research to me. Furthermore, they have taught me to learn always, inculcate a spirit of Learning.

Dr. Sathya Subramani, whose brainchild is the topic undertaken in this thesis, is a person who never fails to surprise me with her undying spirit of inquiry. She helped me take those baby steps into thinking through different aspects of research.

Dr. Soosai Manickam, a jack of all trade, be it making connectors or handling animals. He was always there for the experiment starting early hours of the day and late into the night.

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Dr. Kawin Padmaja, her gentle and sensitive personality, always ready to lend a helping hand whenever I needed it. Dr. Srisangeetha (Sri), who is there with valuable advice when I needed it the most. Dr. Aravindhan V (AV), we learned to handle animals together for research. He became the rabbit-guy, and I turned to rats!

Dr. Farhan Adam Mukkadam (Farry), for pushing me to learn to do coding in Matlab along with Dr. Mahatabb Nundy (Matty), for help in many small and big ways. Dr. Neetu, who herself is an expert in handling animals, helped a ton at crucial moments. Dr. Elizabeth Vinod and Dr. Upasana, whose experienced opinions were of immense help. Other faculties (Dr. Silviya, Dr. Vinay, Dr. Solomon, and Dr. Anand) have been an encouragement. A little help at regular intervals go a long way. I want to express my gratitude to Mr. Vijayanand, who promptly got the experimental materials well in advance, and Mr. Sridhar Raghavan and his colleagues, in the department of microbiology, who helped me with key ingredients of the experiments whenever needed. I want to express my gratitude to other fellow postgraduates, Ms. Geetha (secretary), Mr. Selvam and Mr. Natarajan for keeping the department machinery moving with ease.

The CMC fluid research grant for funding the experiment and the department of microbiology, CMC, Vellore for the help needed for the experiments.

Tim Hunt, Nobel Laureate in Physiology, 2001 was asked a "Is creativity important in science?" He answered, "…I'm not actually sure what creativity means … good science has that sort of quality that you saw put one foot in front of the other, and then when that foot is well firmly grounded, you take another footstep and… that doesn't sound terribly creative to me, but I think what does appeal to me much more is a certain playfulness, and I'm not sure that's the same thing. I admire people who do fun things I mean; I think it actually ought to be fun…" Oh! What fun we had in the days of experiments, as we kept making progress one step at a time! Thanks to everyone in the Department of Physiology.

I am thankful to my wife, Ms. Celin, without whose contribution, I would not be able to accomplish this. Her support and friendship have always been there in both good and bad times. Nothing would be possible without the grace of my Lord Jesus Christ, whose overarching presence leads, guides, and brings stability.

Finally, while I thank all those mentioned above for their help and comments, I can confidently ascertain, all errors remain mine. Thank you, for taking the time to read this manuscript.

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CHAPTER 1 ABSTRACT

Title of study – "Creating a septic shock model and observing the effect of noradrenaline."

Authors – S. Thomas[1], S. Manikam[1], V. Aravindhan[1], S. Subramani[1]

Departments and institution – [1]Dept. Of Physiology; CMC, Vellore.

Background – septic shock experiments usually use lipopolysaccharides (endotoxemia model) or polymicrobial model (using caecal ligation or puncture methods), while researchers rarely use live bacterial injections. Our broader aim is to create animal models of septic shock using different strains of live bacteria to understand sepsis pathophysiology better.

Objectives

A. Creation of a rat model of septic shock using live strains of Escherichia coli and studying the various cardiovascular parameters.

B. To study the effect of administering Noradrenaline (a sympathomimetic drug).

Methods – After random selection, we anesthetized the Wistar rats (WR) for Intra-arterial pressure recordings from the carotid artery using ADInstruments® data acquisition device. Live E-Coli suspended in normal saline (5Mcfarland concentration; dose - 650uL/100g) was injected through the tail vein to induce sepsis and observed for the development of shock (i.e., a drop in mean arterial pressure (MAP) of 50% from the initial MAP). The

first group (n=7), consisted of the addition of *E. coli* alone, whereas the second group (n=7), comprised of rats treated with Noradrenaline. A control group (n=4), was also included in the study. The recordings were analyzed using a customized Matlab® code written by us.

Results – 75% rats (n=14, Group 1 & 2) had a pressure drop in about 2.5 hours (max – 4.2 hrs & min – 0.6hrs) after the addition of *E. coli* and 75% rats (n=7, group 1) died within 57 minutes (max – 62.3 minutes & min – 9.9 minutes) without any intervention. Addition of noradrenaline subsequent to pressure drop after Ecoli, in 7 rats (group 2) prolonged the time to death significantly to about 170 minutes (max - 496.04 minutes & min - 104.47minutes).

Conclusion –

1. The above rat model of E. coli septic shock is useful to study various aspects of septic shock.
2. Noradrenaline prolonged the life of the animal in septic shock.
3. The above model paves the way for sepsis studies due to other common agents of sepsis encountered in clinics.

KEYWORDS: septic shock, animal model, Escherichia coli, Noradrenaline

CHAPTER 2 INTRODUCTION

There is no telling how much back-and-forth exchange happens between the translation of basic science research and its clinical application. Hopes, in this field, are like the graphs on the stock exchange market, at often regular intervals bleak, sometimes blithe, and some sporadic moments of bliss. Moreover, there is a growing sense of this disconnect between pre-clinical research and applicable clinical outcomes (1). It is a skewed mind, to lay the whole responsibility of the lack of translatable findings at the feet of basic science research. Aristotle coined the phrase that the whole is always greater than the sum of its parts. When conclusions drawn from studying the various parts of a complex problem are available, they must undergo another intermediate step that delineates all the challenges of translation. Translational research builds-up knowledge at varying levels to reach the place and point of impact. Yes, it is a tedious and lengthy process, but undoubtedly one that will achieve its purpose.

Animal models have been a pillar in many of the pre-clinical research, though many have raised issues with the gross mismatch of such models and the aspect of ethics of animal use (2–6). However, proper scientifically justified use of animals (7) remains a pivotal step in bridging the gap (2,6,8,9). In this study, we proposed to study the septic shock component of severe sepsis and study the cardiovascular parameters. Taking into account the gleanings from more than century-long experimentation with different animal species, we have gone on to develop a stable and acute model of septic shock.

In the pages that follow, one will find a brief review of the literature on sepsis and various animal models of septic shock used in the past. Then we move on to the details of all the materials and methods employed in our lab to create the model. Following that, results from the experiments are displayed and discussed; drawing it to a close with conclusions deduced from the study results and allusions to the prospects of such a model.

CHAPTER 3 AIM AND OBJECTIVES

AIM:

To creating a septic shock model in adult Wistar rats using *Escherichia coli* and observing the effect of noradrenaline.

OBJECTIVES:

A. Creation of a rat model of septic shock using live strains of Escherichia coli and studying the various cardiovascular parameters.

B. To study the effect of administering Noradrenaline (a sympathomimetic drug).

CHAPTER 4 REVIEW OF LITERATURE

4.1. The relevance of sepsis studies

The disease burden contributed by sepsis is staggering. In a 2016 paper that attempted to quantify the incidence and mortality of sepsis, "global estimates of 31.5 million sepsis and 19.4 million severe sepsis cases, with potentially 5.3 million deaths annually"(10) were put forth. These are crude estimates by far, nevertheless useful. Also, the hindrances to making a positive impact on the global burden of sepsis continue to be the difficulty in quantifying attributable morbidity and mortality, economic disparity, and the under-resourced and low-resilience public and critical health care (11). These barriers are matters regarding the administrative aspect of the disease. However, an even more significant obstacle in combating sepsis is the pathophysiological aspects of it. The effects are so vast that defining and categorizing different stages of sepsis proved to be crucial. After decades of struggle The Third International Consensus definition for sepsis and septic shock (Sepsis-3) was laid down in 2016. Though it is a step in the right direction, a major breakthrough needs to be made in our understanding of the pathophysiology of the disease process, which is further complicated by the differences in the pathobiology of various organisms involved. Moreover, treatment for sepsis has been studded with controversy over the use of corticosteroids, fluid resuscitation and vasopressors agents (12). Antimicrobial resistance jeopardizes management of sepsis and hence requires an epidemiological understanding of such resistance pattern in the

local setting. Hence, to tackle the various dimensions of sepsis, it is necessary to have a proper strategy for understanding the processes involved in the disease.

4.2. The major causative agents:

Some of the most common organisms causing sepsis and septic shock are the gram-negative bacilli Escherichia Coli. Among the gram-positive bacteria, it is the Staphylococcus and the Pneumococcus (13). The sources of these infections could be either urinary tract infections, Pneumoniae, peritonitis, surgeries, and transplant devices. Since this study utilizes *E. coli*, a short word about this organism needs mentioning.

4.3. A brief history of E. coli:

E. coli was first discovered by a German microbiologist and pediatrician in 1884, named Theodor Escherich. He noticed that it is rod-shaped, about 2-3 µm in length and about 1µm wide (14). The bacilli are known for their motility due to either the presence of a single flagellum (helical rotating

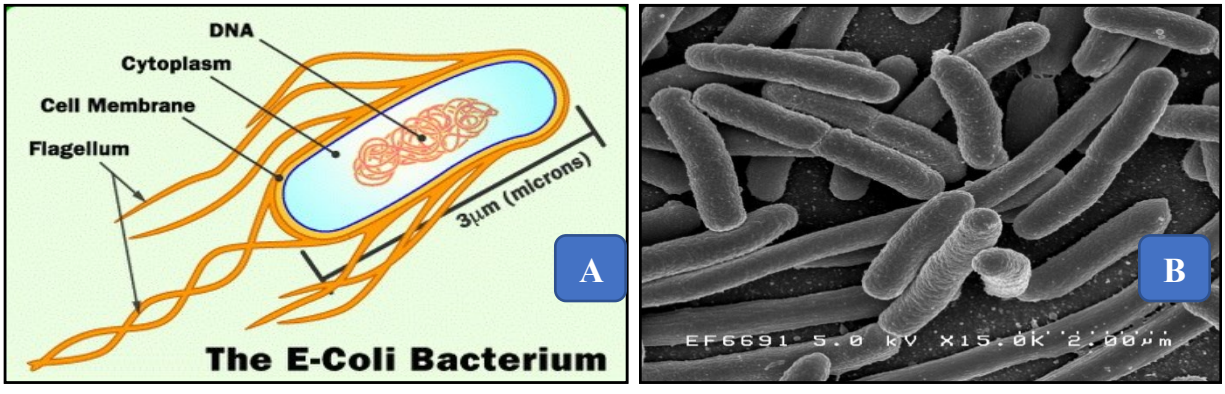

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FIGURE 1: Escherichia coli. A. the parts of the organism; B. The Scanning electron micrograph picture of Escherichia coli, grown in culture.

filaments) or multiple flagella over the cell membrane, as shown in **FIGURE 1**. The presence of flagella makes them robust swimmers, technically called bacterial chemotaxis (15). Several strains have been identified since its discovery, and most of them being non-pathogenic. It is a normal commensal of the intestine of humans and animals. Within a few hours after delivery, it colonizes the gut of humans, albeit, many strains of *E. coli* have managed to transform into disease-causing ones (16). Most notorious are those that cause gastroenteritis (diarrhea), urinary tract infection, and neonatal meningitis (17). Shiga toxin-producing *E. coli* (STEC), also called the Enterohemorrhagic *E. coli* (EHEC), is the most commonly reported pathotypes associated with foodborne outbreaks that are easily destroyed by properly cooking food (18). Other serotypes include, enteropathogenic *E. coli* (EPEC), entero-toxigenic *E. coli* (ETEC) also known as traveler's diarrhea, enteroaggregative *E. coli* (EAEC). O26:H11, O91:H21 and several other serotypes of STEC/EHEC have been found to cause disease in humans (19,20). Here, 'O' (somatic antigen) determines the serogroup, 'H' (flagellar antigen) determines the associated H antigens and a 'K' antigen that is the capsular surface antigen (19,20).

Pathogenicity is made manifest when *E. coli* has acquiesced one or multiple virulence factors during bacteremia (17). The virulence factors that contribute to this are as follows (21):

- Genetic attributes (genetic exchange by transduction and conjugation, transmissible plasmids, pathogenicity islands)

- Toxins (heat-labile (LT) toxin, heat-stable (ST) toxin, Shiga toxin, endotoxin – Lipopolysaccharides). The presence of capsules prevents the effectiveness of antimicrobials.
- Adhesins (Type 1 fimbriae, P fimbriae, S fimbriae, EPEC adherence factor)
- Invasins (hemolysin, Shigella-like "invasins" for entering cells and dissemination).
- Motility or chemotaxis using the flagella.
- The cell wall is the target of many antimicrobials. Conversion of the bacteria to an L-form, a wall-deficient state, renders the wall-targeting antimicrobial agent completely useless.
- Biofilms are the invisibility cloaks of the bacteria from naïve macrophages and make them resistant to antibiotics as well. Biofilms are also known as sessile aggregates. The other type of growth is known as planktonic cells.
- In 1673, Antoni van Leeuwenhoek described a planktonic micro-organism. Bacteria may roam around the body in the bloodstream as a planktonic and are known to be rapidly dividing. They exhibit four stages: i) lag phase; ii) exponential (logarithmic phase); iii) stationary phase; iv) death phase. *E. coli* is an example of a single-celled planktonic bacteria. A triboelectric charge which develops during their flow in the bloodstream inhibits their metabolism. The charge is the result of constant friction with cells and the vessel wall. Since they are

inactive the effectiveness of the antibiotics in the bloodstream is thwarted. These bacterias can then enter the RBCs by the action of hemolysin that they produce. Inside the RBC, they proliferate unhindered (13). The various types of bacterial forms and degrees of sepsis it causes are mentioned in TABLE 1.

***Table 1*: types of sepsis and the bacterial forms of sepsis-causing bacteria**

Bacterial form	Types of sepsis
Planktonic Sepsis	Severe sepsis
	Septic shock
	Fulminant
	sepsis
Encapsulated	Subacute
	sepsis
	Chronic sepsis
Biofilm	Relapsing sepsis
L-form	Indolent sepsis
	Latent sepsis
	Dormant sepsis

E. coli's contribution to biology and medical science and industry: Many advances in fields like medicine, biology, pharmaceuticals, etc. have been made using *E. coli*. These are enlisted below (22). a. Biotechnology and Genetic Engineering: molecular cloning, rDNA. b. Biology: studying genetic code, structure and function of ATP synthase, and discovery of restriction enzyme. Genetic sequencing unravels *E. coli*'s fantastic plasticity and dynamism, which produces its genetic and phenotypic diversity. c. Pharma: development of new recombinant therapeutic protein.

4.4. Pathogenesis of sepsis

Colonization of the host mucosa, inducing host damage, evading its defense mechanisms, and rapid multiplication are some of the tactics *E. coli* uses for infecting (20). *E. coli* sepsis created in baboons (23) done by F B. Taylor Jr. and S.D. Kosanke, consisted of infusion of *E. coli* (4x 10^{10} organisms/kg) over 2hrs. They divided the response into four stages:

- Stage I – inflammation

- Stage II – coagulation,

- Stage III – Cell injury,

- Stage IV – Cell degeneration.

4.4.A) Stage I - Inflammation

The potent antigens on the cell surface produce a severe inflammatory response called systemic inflammatory response syndrome (SIRS), which is due to the innate immunity of the body. Tumor necrosis factor-α (TNF- α)

interleukin-1 (IL-1) and interleukin-6 (IL-6) are the major pro-inflammatory circulating cytokines during SIRS (13,24,25). The explosive increase in cytokine concentration is also known as a "cytokine storm." Pattern recognizing receptors on the antigen-presenting cells, for example, the toll-like receptors (TLR) on the surface of the macrophages, are activated by the attachment of these endotoxins. Endotoxins or any other antigens on the surface of the invading bacteria act as a pathogen-associated molecular pattern (PAMP) or danger-associated molecular patterns (DAMP). These different molecular patterns are identified by pattern recognition receptors like the TLRs. There is a decline in the amount of WBC's in circulation as these adhere to the endothelial cells. TNF activates these neutrophils that get released from the macrophages. The severity of these tissue-specific and systemic events is determined by several factors mentioned above.

4.4.B) Stage II – coagulation

These mediators have a very well documented effect of starting coagulopathy primarily by the expression of tissue factor (TF) on macrophages and endothelial cells (24,26), causing disseminated intravascular coagulation (27,28). This is evidenced by the low fibrinogen levels as it is consumed for the coagulant response. DIC is the result of a massive counterattack on the part of the animal to ward of the pathogens and not a failure of its defense mechanism.

4.4.C) Stage III & IV – Cell injury and degeneration

In this stage, there is the rise of cell injury markers such as SGPT, extravasation of fluid leading to edema, and subendothelial tissue degeneration. Endotoxin shock results from the sequestration of the neutrophils and platelets, which in turn make the endothelium sticky and leaky with peripheral vasodilation, thereby decreasing the total peripheral vascular resistance attributable to the release of endothelium-derived relaxing factor (EDRF) as nitric oxide (NO) (29,30), subsequently progressing towards to multi-organ failure (see FIGURE 2).

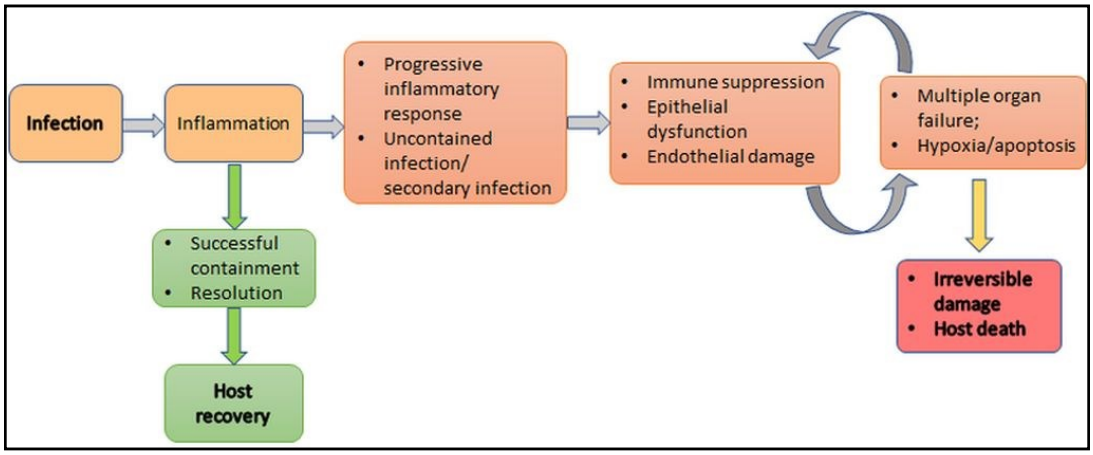


***FIGURE 2*: Pathogenesis of sepsis.**

4.5. History of studies on sepsis

Humankind's battle with microbial agents is from antiquity, known to us by the Egyptian writings on the pyramids about the money spent on foods of the onion variety to ward off small insects that were associated with causing diseases (31), as well as the plagues due to swarm of flies that caused destruction mentioned in the old testament. A Roman scholar, Marcus Terentius Varro (116–27 BCE), is known to have recognized the importance of tiny creatures in the pathogenesis of the disease. He writes, "Precautions

must also be taken in the neighborhood of swamps, both for the reasons given, and because there are bred certain minute creatures which cannot be seen by the eyes, which float in the air and enter the body through the mouth and nose and there cause serious diseases. “What can I do”, asked Fundanius, “to prevent disease if I should inherit a farm of that kind?” “Even I can answer that question”, replied Agrius; “sell it for the highest cash price; or if you cannot sell it, abandon it.” (31) Well, a better solution was being provided once humankind started understanding the basis of these diseases. A huge leap was made when Anthony van Leeuwenhoek (1632–1723) first described “animacules” in 1674, which opened the way for other scientists to develop the germ theory further. A crowning achievement was made when Louis Pasteur was able to prove the germ theory of disease. Ever since the understanding of the relationship of germs and disease, it has made significant improvement by implementing protocols – from hand wash to use of sterile methods of surgery; and remedies to infections – vaccines (as a handy preventive measure) and antibiotics, were increasingly applied to bring drastic changes in our practice of medicine. However, the fight with sepsis can hardly be said to be over. There are dimensions of this phenomenon that kept man searching for answers.

Animal models became very important in the quest to understand the disease process. For example, Richard Pfeiffer (1858–1955) trying to understand cholera pathogenesis using Vibrio cholerae, was unable to find the typical exotoxin because until that time, owing to the great work by Ludwig Brieger

(1849–1919) on Diphtheria in which he was able to isolate the exotoxins from the supernatant of the cultures with live organisms. Diphtheria and other organisms were, until Pfeiffer, found to have heat-labile exotoxins. Pfeiffer inoculated guinea pigs with V. cholerae that were immunized and still found that the guinea pigs died. He concluded that this was not due to a classic heat-labile exotoxin protein that was produced during bacterial growth; instead this was a heat-stable substance released from within the bacteria itself after the cell death. Endotoxin was the name he gave to this substance since this was produced within the bacteria. With the discovery of the endotoxin, many investigators tried to replicate reproducible and straightforward models of endotoxin mediated shock in laboratory animals (32).

4.6. Various sepsis models:

Three categories have been put forth as animal sepsis models. A) The administration of exogenous toxins like lipopolysaccharides. B) Exogenous injection of bacteria into the animal either through the intravenous or the intraperitoneal route. C) Disruption of the host barrier, for example, colon ascendens stent peritonitis (CASP) and caecal ligation and puncture (CLP) (33). Another method suggested early on also included an introduction of material that is infected with the organism into the subcutaneous soft tissue to produce abscess (32). Several humanized mice models using genetic engineering methods are being employed to overcome the limitations caused by differences in genes caused by evolutionary divergence (34).

4.6.A) Toxemia Models:

Toxaemia models have been to date the most commonly used method for sepsis studies in animal models. This is because it is easy to store the endotoxins in lyophilized form, and an accurate dose of the endotoxin can be measured and administered. A comparison between this model and those that happen in humans are mentioned in TABLE 2. Several chemical agents, such as lipopolysaccharides, lipoteichoic acid, zymosan, peptidoglycans, and others have been used in the past (35). Borden and Hall (36) and Braude et al. (37) were among the early investigators who proposed the association of septic shock due to gram-negative bacteria with bacterial endotoxins even though the picture of *E. coli* infection did not match the features seen in animals that were given *E. coli* toxins (38). Later with the advent of purified endotoxins, which were mainly administered into dogs during the early era of experimentation, it was well documented all the observations from these experiments. Initially it produced excessive salivation and normal bowel movements followed by vomiting and diarrhea. The dog usually died within four to twenty-four hours from the point of administration (39). Many drugs were used as intervention in this model, as the use of corticosteroids and anti-TNFα. Though they were compelling, they had reduced translational benefit (40).

***Table 2*: Comparison of the toxemia model in animals with human sepsis**

Similarities	Differences

• Endotoxin induces shock state in both mice and humans.	• Humans are more sensitive to endotoxin. • The model does not recapitulate the clinical hemodynamic phases. • Different cytokine responses.

4.6.B) Intravascular infusion of live bacteria:

69% of septic shock cases, 25% of life-threatening sepsis, and about 17% of patients with sepsis have turned positive for blood cultures, demonstrating a positive correlation between the chance of finding a positive blood culture with an increase in the severity of sepsis (35,40). Positive culture meant the presence of live organisms in the bloodstream and directly affected the severity of the disease progression. The most common organism used for this purpose has been *E. coli*. An exaggerated TNFα response is seen along with a decline in blood pressure and early death (41), which improved with pre-administering TNFα inhibitor. Fulminant DIC is due to complex and massive inflammatory response leading to multi-organ damage (MODS) (42).

***Table 3*: Comparison of the Live-bacterial infection model in animals with human sepsis.**

Similarities	Differences

• Induces a similar shock state that is observed in human bacterial infections. • This model is far more accurate in replicating clinical infection than an artificial LPS administration approach. • Dosage and the viable phases of bacteria inoculant can be manipulated to mimic clinical infection, sepsis, or SIRS in mice induces shock state in both mice and humans.	• The high inoculating bacterial dose is usually needed. • Pathologic phenotypes observed in models are likely due to endotoxemia and not an infection.

In the porcine model, both gram-negative and gram-positive bacteria were used, resulting in acute respiratory failure and shock. In another study, P. aueroginosa was used resulting in hypotension (43). In study done on sheep, the following features were noted after a sub-lethal dose of *E. coli*; it produced hypotension, increased heart rate, and respiratory rate, decreased urine output and hyperlactatemia (44). In dogs, *E. coli* injections produced profound hypotension with low cardiac output and hypoperfusion of the splanchnic circulation which was not amenable to fluid resuscitation. Splanchnic circulation was more affected than the systemic circulation

(45,46). The primary argument against the use of this model is that it causes early death due to cardiovascular collapse, which is rare to see in human sepsis.

4.6.C) Peritonitis model:

Ahrenholz and Simmons, in 1980, used an intraperitoneal injection of viable *E. coli* ($2x10^8$) into rats (47). The *E. coli* was suspended in saline solution, and this produced 100% mortality, whereas when introduced using *E. coli* in bovine fibrin clot, it prevented early death. This was most likely due to fibrin preventing the absorption of the *E. coli* from entering the systemic circulation. In dogs, another model was introduced were they were instrumented and implanted with bovine fibrin clot with viable *E. coli* bacilli (48). This model replicated many features of human sepsis and allowed the investigator to have better control over the dosing. Nakatani et al. used autoclaved rat feces and agar that were then injected with a known quantity of *E. coli* or B. fragilis and introduced into the peritoneum. Results showed a decrease in peripheral vascular resistance, high lactate levels in blood, and increased cardiac output. Guinea pigs were used by Alexander et al., where he placed Alzet osmotic mini pumps in the peritoneal cavity to deliver the required dose of the *E. coli* or Staphylococcus aureus. All these models require daily monitoring of the animals and therefore need more help in maintenance over several days.

Table 4: **Common Sepsis Behavioral Scale Parameters.**

Behavioral Scale Parameters
Weight loss, level of food/water intake
Presence or absence of activity (foraging, eating, etc.) and assessment of ataxia
Quality of bowel movements
Appearance of eyes (open, closed, secretions)
Self-mutilating behavior
Evaluation of respiratory rate and associated noise/gasping
Bleeding from an orifice
Appearance of fur (piloerection, soiling, etc.)
Posture

4.6.D) Fecal peritonitis by Caecal ligation method or surgical perforation:

This method was made known by Wichterman et al. (32) and has been used widely since then. He initially described it in rats, and others have extended it to other species like sheep and mice. In caecal ligation and perforation (CLP), the abdomen is dissected, and the caecal junction pulled out and loaded with feces by milking the stool retrograde from the large intestine towards the caecum. At the ileocecal valve, ligatures were applied, and a small nick is put

over the anti-mesenteric caecal surface two times. The whole of the abdominal contents was then placed back in, and the wall sutured in layers. After they recovered from anesthesia, the rats ate and drank and appeared quite normal for about half a day after which they became increasingly sick. They were assessed on multiple behavioral scales as mentioned in TABLE 4. In their study, only 11 survived out of the 184 animals that underwent CLP (32). This model does not allow for the effect of single organism sepsis but utilizes mixed gut flora.

Furthermore, the whole experiment takes a couple of days to monitor, requiring specialized centers and more manpower. Another disadvantage of this is the inability to regulate the dose of the contamination and thereby the severity of sepsis. However, this model proves worthwhile to study physiological differences within species between those who survive, and those succumb to septic shock. TABLE 5 gives the comparison of the peritonitis model with sepsis in humans.

4.6.E) Colon ascendens stent peritonitis (CASP):

It is a relatively new model that is very similar to CLP. Here also, like CLP, CASP causes polymicrobial infection. The procedure is the same as in CLP but without the ligation of the caecum at the ileocecal junction. A stent is placed in the caecum and sutured into place, such that there is a constant flow of fecal matter into the peritoneal cavity. It is shown that serum cytokines increase from 6 to 18hrs; death generally occurs 1 to 2 days after the surgery.

***Table 5*: Comparison of different Peritonitis models in animals with human sepsis.**

Similarities	Differences
• CLP is considered the gold standard for sepsis research • Mimics human appendicitis or perforated diverticulitis • Shows close resemblance to the hemodynamic, immunological and metabolic stages of a human sepsis.	• Outcomes are variable. • The strains that produce the sepsis may not that present in human sepsis.
• CASP causes a pro-inflammatory profile of clinical sepsis 3 hours after stent placement. • Sepsis induced multi-organ failure is seen. • Greater leak of stool than CLP	• Does not show the different hemodynamic phases of clinical sepsis. • CASP t 0ake lesser time to develop sepsis than CLP

4.6.F) Soft-tissue infection model

In this method, used in 1964 by Albercht and Clowes (49), 10% calcium chloride solution, about 10-20ml, was injected intramuscularly, which resulted in tissue necrosis that invited infection by a lot of pathogens and saprophytes. Another investigator introduced cloth tape soiled with feces into the soft tissue whereas Gahhos et al. compared all three models of sepsis, i.e., IV infusion of live *E. coli* with and without IV saline volume expansion, and thirdly, IM injections of live *E. coli* a day before the experiments, in pigs that were not mature. He found that only the third category developed hyperdynamic effects and concluded that an infectious focus is needed for the development of the hyperdynamic state. Wolfe and Durkot in 1989 showed similar results in guinea pigs, where they compared the metabolic response to infection.

CHAPTER 5 METHODOLOGY AND EXPERIMENT SCHEMA

5.1. Methodology

First, the research proposal was drafted and presented before the institutional review board (IRB). After obtaining the IRB approval (IRB Min NO. 11033 [Other], Dated – 04.12.2017), the experiments were started with the funding provided by the IRB, The details of the materials and methods used are described below.

5.1.A) MATERIALS REQUIRED

1. Anesthetic agents – Inj. Ketamine, Inj. Midazolam
    a. Dosage for Ketamine – 80mg/kg/hr; vial contained 10ml of 50mg/ml of the drug.
    b. Dosage for Midazolam – 1.5mg/kg/hr; vial contained 10ml of 1mg/ml of the drug.
2. Intervention drug – Inj. Noradrenaline. Dosage of NORAD® administration was (0.3 – 0.6 μg/kg/min).
3. Live microbial agent for injection – Escherichia coli suspended in Normal Saline.
4. McFarland standard – It was named after the scientist – JOSEPH McFARLAND, M.D., who developed it and published it first in 1907 (50). It is used to provide an approximate number of bacteria suspended in a liquid. The turbidity of the

test solution is compared with that of McFarland standard. The McFarland standard was made in our lab using the DALYNN biologicals catalog number TM50-TM60 (51), which can be found in the appendix. We made this in the lab by using barium chloride and sulfuric acid. The result of these two chemicals reacting is the production of fine precipitate called barium sulfate. After shaking it on a vortex mixture before examination

Cat No.	McFarland Standard	1% $BaCl_2$ (mL)	1% H_2SO_4 (mL)	Approximate Bacterial Suspension / mL
TM50	0.5	0.05	9.95	1.5×10^8
TM51	1.0	0.10	9.90	3.0×10^8
TM52	2.0	0.20	9.80	6.0×10^8
TM53	3.0	0.3	9.7	9.0×10^8
TM54	4.0	0.4	9.6	1.2×10^9
TM55	5.0	0.5	9.5	1.5×10^9
TM56	6.0	0.6	9.4	1.8×10^9
TM57	7.0	0.7	9.3	2.1×10^9
TM58	8.0	0.8	9.2	2.4×10^9
TM59	9.0	0.9	9.1	2.7×10^9
TM60	10.0	1.0	9.0	3.0×10^9

Figure 3: The McFarland standard made from mixing barium chloride and sulphuric acid in varying proportions to give rise to different standards (from 0.5 to 10). The last column also suggests the approximate bacterial load in a solution that matches a standard.

of the turbidity, that is the absorbance or the light intensity is measured and compared with the test solution. As the concentration of Barium chloride increases, the amount of absorbance (also called turbidimetry) or the intensity of light (also called nephelometry) varies proportionately. Many have used this standard for various comparisons (50,52,53). The table below shows the different standards and their concentrations, as well as the approximate bacterial suspension. Figure 3 shows the preparation of the standard solution in our laboratory. (see appendix attachment for details); FIGURE 4 shows our lab preparation of the McFarland standard.

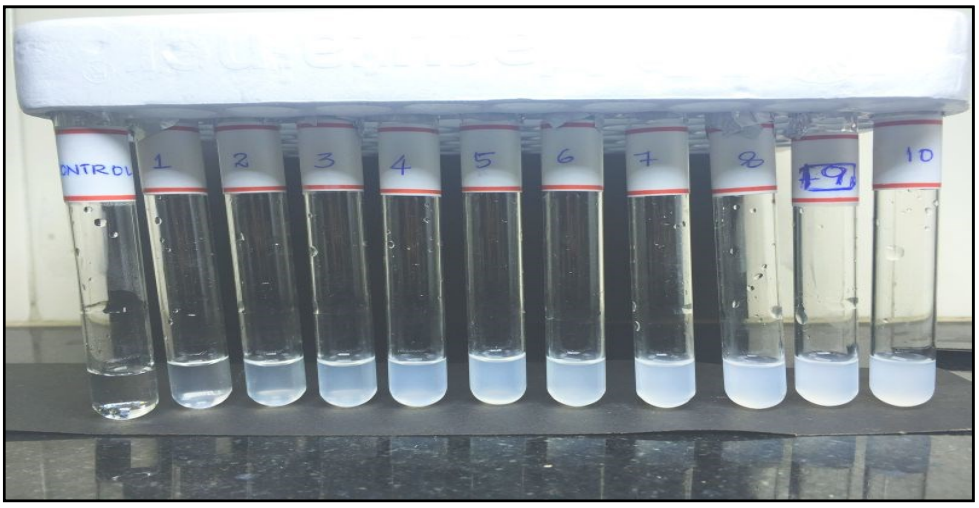


Figure 4: The McFarland standard. We added a control with pure distilled water.

5. Inj. Heparin (5000 IU/mL; 10mL vial); Dose used is 3 – 6 IU/kg/hr. A dose of 40 µL from the vial was added to the Normal saline inside the pressure bag.

6. Inj. Hep-Lock (Heparin Lock Flush-Injection)
7. Normal saline 500ml & 100ml.
8. 24G or 26G IV cannula, 3-way stop cork.
9. 1cc, 5cc, and 20cc syringes.
10. Two Infusion pumps for drug delivery.
11. Surgical instruments (scissors, forceps, spatula, Bulldog clamp)
12. Thread, connectors.
13. Oxygen cylinder, mask.
14. iPex Pressure Monitoring kit® - developed by B L Lifesciences Pvt. Ltd.
15. For calibration of the pressure transducer – An aneroid barometer.
16. ADinstruments® Data acquisition system.
17. A Laptop with LabChart data analysis software® for the recording of data.
18. UPS – for power backup.
19. Antiseptic solution (70% Ethyl alcohol)
20. Cotton, gauze
21. Dakin's Solution
22. RESPIRATORY BELT – to record the respiratory movements of the animals, a new respiratory belt was made in house. The details of the respiratory belt are given in the paper (to be published) as an e-journal later. This belt was fastened across

the chest wall of the rat to monitor respiratory efforts throughout the experiment.

5.1.B) Animal selection:

In this study, the animal model was made using adult Wistar rats selected randomly irrespective of their gender. The weight of the rats ranged from 250grams to 350grams. The animals were provided by the animal house of Christian Medical College, Bagayam, Vellore, after obtaining ethical clearance from the Institutional Animal Ethics Committee (IAEC approval number – 8/2018; Dated – 26.02.2018).

The animals were included under two primary arms – **the test group** (E. coli only group); **the intervention group** (E. coli + Noradrenaline); and a **control group** where only normal saline was injected, was also included in the study.

The significant steps of the whole experiments were as following:

A. Tail vein cannulation for maintenance of the animal under a good plane of anesthesia throughout the whole duration of the experiment.
B. Procuring live *E. coli* bacilli cultured on Agar plates from the Department of microbiology. Making a solution of *E. coli* bacilli in normal Saline and making it to the desired concentration of 5 McFarland units.
C. Dissecting out the Carotid artery in the neck and inserting a fluid-filled catheter connected to the iPex pressure transducer for intra-arterial pressure recordings.

5.1.C) Anesthesia – tail vein cannulation.

Anatomy of the tail vein: The vertebral column of the rat continues into the tail as the tail vertebra, which forms the innermost core. Surrounding the innermost bone-core are the tendons and dorsal coccygeal muscles that are in turn, covered by skin. The skin and the tendons are filled in between with subcutaneous tissue through which the neurovascular structures run their course. The rat has two prominent veins situated dorsally and laterally to the tail vertebra's transverse process, on both the right and left side. These are large as compared to the others and can be localized subcutaneously, maintaining their luminal diameter without any significant change till the tip

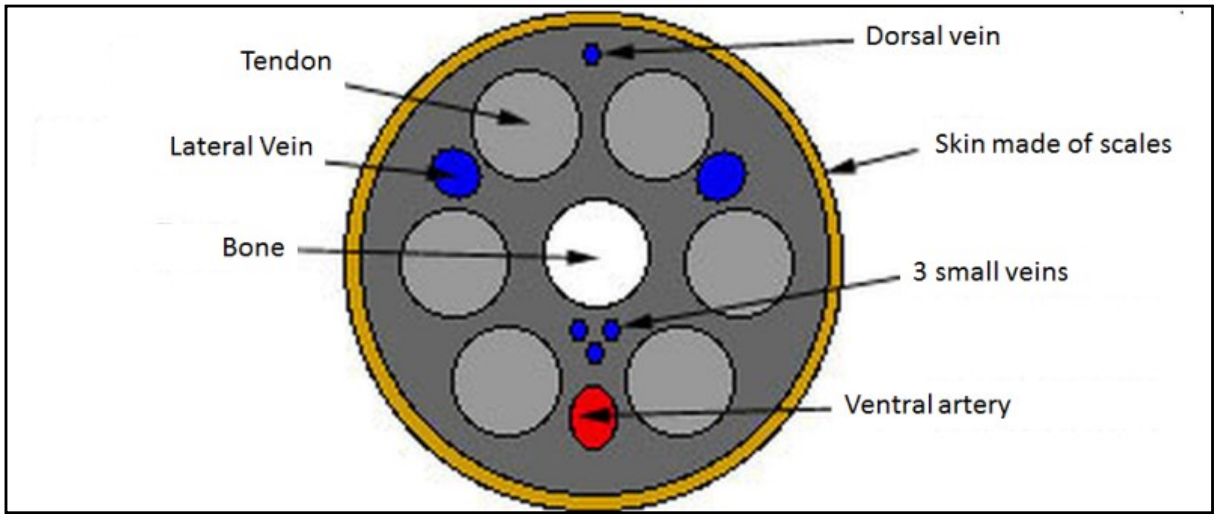


Figure 5: **The cross-section of the rat tail vein showing a layout of the major artery and veins supplying the tail. The two lateral veins on either side of the transverse process of the tail vertebrae are the best place for**

(54–56). Besides, there are other veins, such as a small dorsal vein and three small veins situated ventral to the bone. A large artery is situated in the ventral aspect of the tail.

Procedure of tail vein cannulation: The method of tail vein cannulation is like

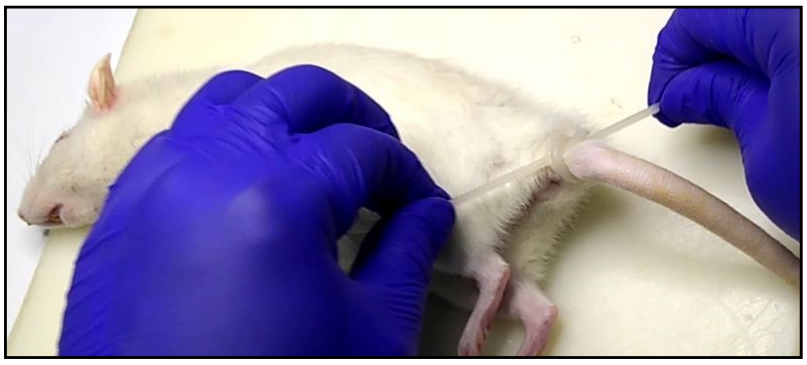


***Figure 6*: Tying a tourniquet to obtain an engorged vein for better identification and easy cannulation.**

any other IV cannulation done in clinical practice with the help of an IV catheter. Here, we used a 24G BD Insyte® to insert it into one of the lateral veins. After the rat is put under anesthesia (the method of induction of anesthesia is given under section **5.1.E (i)**), the animal is made to lie on a plane surface. The scales on the tails are scraped off and wiped clean before the start of the cannulation. Make sure that the sterile 24G catheter is

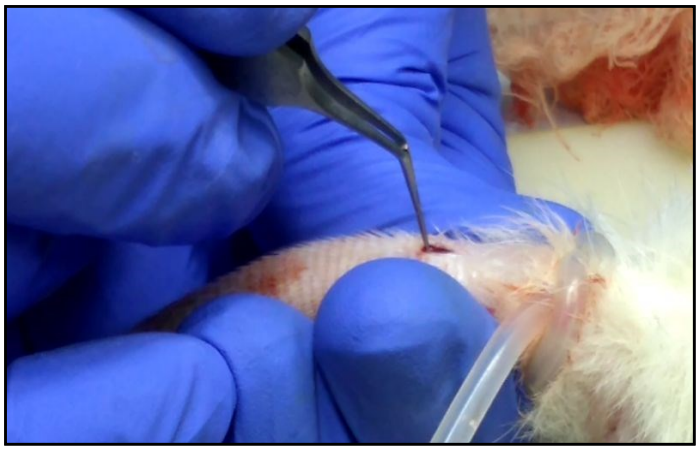


***Figure 7*: Tunneling gently along the course of the vein parallel to it to free it off its fascial connections. This produces a bigger bulge.**

removed from the covering and flushed with heparin lock flush solution and

kept ready along with a #11 scalpel blade on to the holder. Where the tail

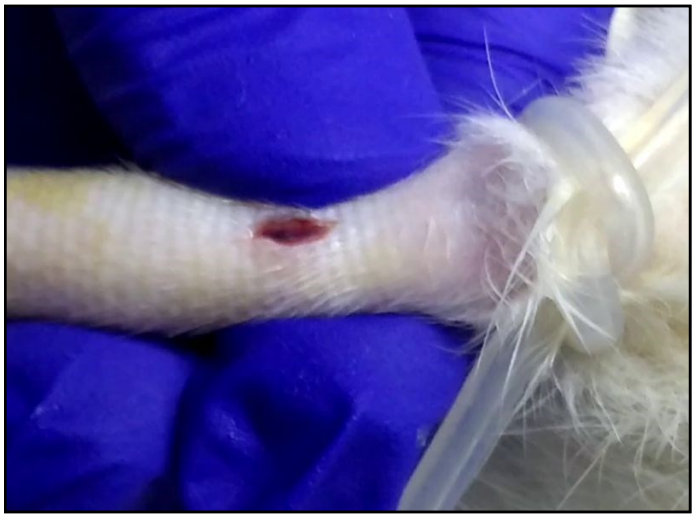


***Figure 5*: A clearly visible lateral tail vein that is devoid of fascial attachments. It is made taut by pulling the tail.**

begins, a tourniquet (figure 6) is tied tight enough to make the veins engorge, which makes it visible from the surface. Using the surgical blade, make a straight 0.5cm superficial incision carefully on the skin just above the vein or slightly parallel to it about 2cms below the tourniquet. Pinch on the lateral side of the incision in such a way that the wound gapes. By using blunt curved forceps to tunnel (figure 7) along the path of the vein, it becomes

FIGURE *6*: A straight bulging lateral vein ready for cannulation. Traction at the tail end gives the proper counter pressure for easy.

bulged and clean to make cannulation easier (figure 8 & 9). Now, using a 24G cannula that has been adequately flushed with Hep-lock solution, puncture into the vein while continually maintaining a gentle traction on the tail with the other hand or with the help of the assistant.

The traction is necessary to keep the vein taut enough to produce resistance for the smooth entry of the cannula without the vein getting kinked. When inside the vein lumen as understood by the filling of the flashback chamber, push the catheter alone so that it goes further into the lumen of the vein, and the needle is drawn out. Without disturbing the cannula, loosen the tourniquet from the tail and push the cannula further. Now using a 1ml syringe and the catheter needle, fill the space inside the hub with the hep-lock solution till the edge and push some saline through the catheter to ensure that the line is

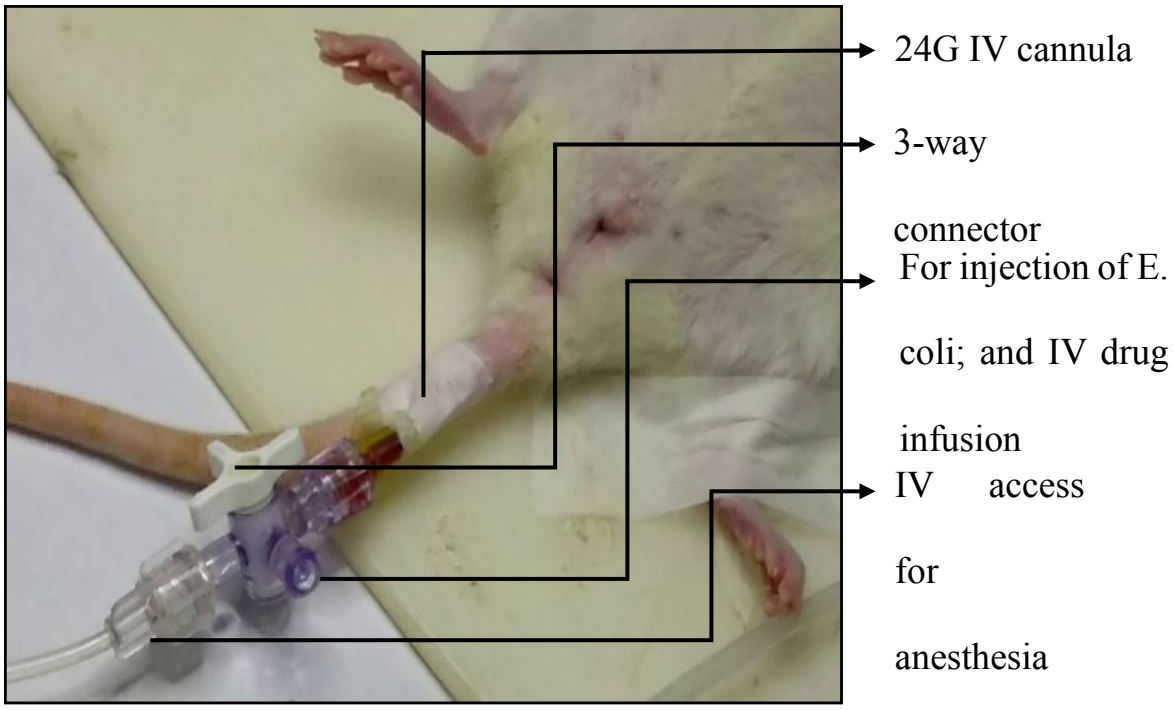


***Figure 7*: A tail vein cannulated, and catheter relates to a 3-way connector that allows for maintenance anesthesia and administration of E.coli and drugs.**

patent. Secure the catheter in its place by using micropore stickers cut into thin long enough strips to wrap the tail and the hub and wings of the catheter rendering it fastened to the tail of the animal. Connect the three-way cannula to the 24G catheter after priming it with a hep-lock solution so that air bubbles are prevented from entering into the vascular system. This is then connected to the infusion pump line that is loaded with the anesthetic calculated beforehand according to the weight of the animal (figure 10).

5.1.D) Preparation of the E. coli.

(i) *Making an E. coli inoculum:* Agar plates that had live cultures of the pure strain of E. coli were obtained from the microbiology department a day before the experiment and stored in a separate container at 8℃. On the day of the experiment, the agar plate is kept out for some time to allow it to reach

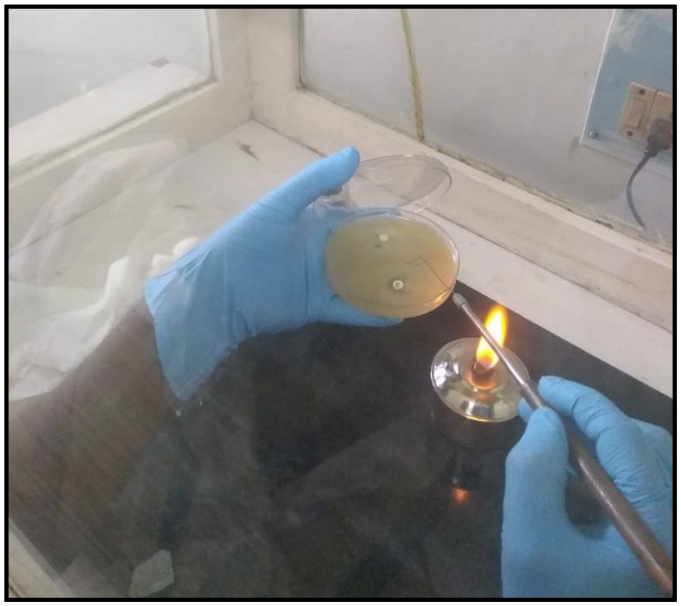


***Figure 8*: An agar plate with live E. coli. Using the nichrome wire loop technique to scrape out some colony of bacilli from the culture plate and mix it in the sterile saline solution.**

room temperature in a bio-safety hood. A nichrome wire inoculating loop (figure 11) was sterilized by flame. 2ml of sterile saline is taken from the 100ml NS bottle and aliquoted into a sterile glass test tube. Care must be taken to avoid contamination of saline or the agar plate with other organisms due to faulty technique. Once the nichrome loop cools down, the organism is scraped off the agar surface twice, and the *E. coli* is mixed into the saline and shaken until it becomes turbid (figure 12). This test tube is then compared with the McFarland tubes to match the standard #5. Once the turbidity matches, close the test tube with a cotton plug and again expose the nichrome loop to flame until red hot and set it aside after cooling down.

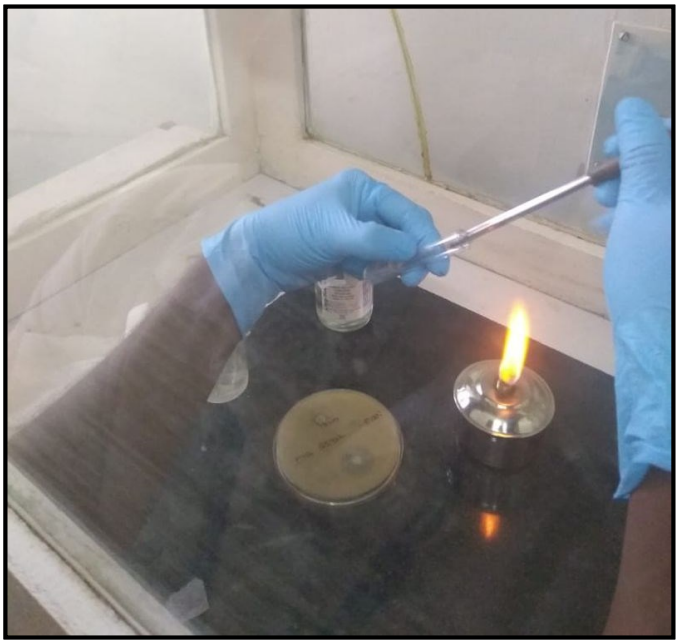


***Figure 9*: Scraped out colonies of bacilli from the culture plate are mixed in a sterile saline solution to the degree that the turbidity of the solution matched the 5 McFarland units.**

(ii) *Dosing*: Now, the 5 McFarland unit turbid saline *E. coli* broth was used to inject the required quantity into the rat intravenously through the

three-way cannula connected to the rat tail vein catheter. Standardization experiments done previously, helped in fixing a dose of 650 µL/100g. Therefore, for a 275g Wistar rat, the amount of *E. coli*-NS suspension was = $\frac{275*650}{100*1000}$, i.e., 1.8 mL. The 3-way connector in the tail vein catheter is at first filled with NS till the brim. 1.8mL was taken in a 2cc syringe and connected to this other inlet. The anesthetic pump was stopped momentarily, and the 3-way was turned such that the other entrance site was online, and the anesthetic line was offline with the rat tail vein. Gently push the saline broth solution into the tail vein. There was an immediate drop in pressure, which is due to the sudden bolus effect of the anesthetic rush into the system. Stop pushing and allow the effect to wear off. Do this a couple more times till all the anesthetic in the catheter completely moves into the circulation. Now the required dose of *E. coli* can be given at a steady slow pace taking approximately 3-4minutes.

(iii) *Precautions while handling E. coli*: Proper personal protective measures must be maintained. Wipe and spray the area with 70% ethanol in case of spillage on the table. Spray the syringe and the test with 70% ethanol after use and then dispose of according to the bio-safety mandates. The test tubes and syringes are additionally soaked in Dakin's Solution (Sodium Hypochlorite) (57).

5.1.E) Carotid artery cannulation

i. *Induction of anesthesia*: The rodent family is generally considered to lack the ability to vomit, especially rats (58,59). Hence the need to keep the animal for overnight fasting is not necessary. After first weighing the rat, the dosage of both Ketamine (80mg/kg/hr) and Midazolam (1.5mg/kg/hr) is

***Figure 10*: Handling the rat using a towel to cover the head and body, then holding the head in-between the index and middle fingers. Throughout the whole procedure the tail must be firmly held by the other hand.**

calculated. For example, if the rat weighs 275 grams; the dose of Ketamine would be = $\frac{275*80}{1000}$ mg/hr, i.e. 22.0 mg/hr. Now the vial contains 50mg/ml of Ketamine, therefore to get 22 mg of the drug, we will take $\frac{22}{50}$ ml i.e., 0.44 ml of the drug. Similarly, Midazolam dose = $\frac{275*1.5}{1000}$ mg/hr, 0.41ml of midazolam from the vial is drawn (Midazolam vial contains 1mg/ml). Hold the rat by its tail and move it out of its cage on to a flat surface. Without leaving its tail, as the rat tries to moves forward, hold the head end of the rat with a piece of

cloth with forked fingers such that the head is between the index and the

Figure 11: **Induction of anesthesia using the cocktail mixture of Ketamine and Midazolam already loaded in a 1mL syringe is given intraperitoneally. The animal is held at a slightly slant position.**

middle finger and the other fingers gently curl around the thorax and upper limb (See FIGURE 13). Do not squeeze the animal too tight as it will start to struggle because of discomfort. Curl the tail around the little finger. If an assistant is available, then he/she can hold the animal with both hands, and you can administer the dose. So, in this case, 0.44 ml of Ketamine and 0.41 ml of Midazolam are loaded together into an insulin syringe and injected intra-peritoneally, as shown in the figure. Hold the rat in a slightly head-down position so that the peritoneum with the bowels is pushed down. The sweet spot is between the inguinal region and the midline, go in perpendicularly and aspirate before injecting the drug into the peritoneal cavity (See FIGURE 14). Now hold the rat upright and leave it back in its cage and wait for the

anesthetic to set in. It takes about 10-15 minutes for the rat to be under anesthetic. The depth of anesthetic can be assessed by pinching the skin or the toe. The next step was to perform rat tail vein cannulation, details of which are given above.

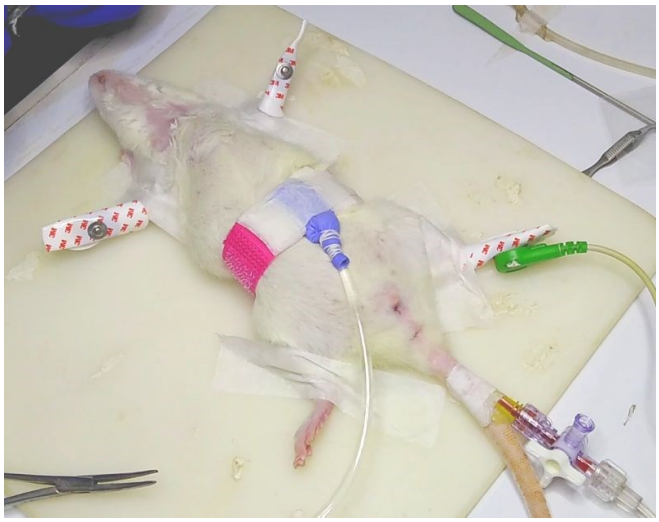


***Figure 12*: The tail vein cannulated animal is under a good plane of anesthesia. ECG electrodes are applied to the two upper limbs and left lower limb. The respiratory belt is snugly fit around the thoraco-abdominal region.**

ii. *Maintenance of anesthetic*: The whole experiment goes on smoothly if the plane of anesthetic is proper throughout the experiment. Hence it is crucial to get the tail vein cannulation right. The procedure for the same is described in detail above (see section **5.1.C**). Since the loading of the drug is crucial, we will now discuss the calculation of the dosing of the drugs used. The assumption was to load the drugs and IV fluids required to sustain for about 8 hours. It was decided to load for 10 hours to account for the volume of fluid in the tubing and residue in the syringe. Following our example above, for 10 hours 4.4 ml of Ketamine (200 mg) and 4.1 ml (4.1

mg) of Midazolam is added into a 20 ml syringe. Fluid should be replaced @ 10 ml/kg/hr, that amounts to [= $\frac{10*275}{1000}$], 2.75 ml/hr. We take into account the volume of fluids given as drugs, flush from the transducer, *E. coli*-saline mixture (~1.75 ml), 1 ml/hr of NS was added to the 20cc syringe loaded with the anesthetic. So, the whole syringe contained 18.5 ml of fluid. Place the syringe on the syringe pump and set the flow of the pump to about 1.8 ml/hr (maximum flow rate for the current example) and prime the tubing with drug before connecting it to the tail vein 24G catheter. Priming must never be done with the tube connected to the IV cannula; if air is present, it can push the air in causing an air embolism, and if there is drug, then sudden rush of a bolus dose of anesthetic results in the sudden death of the rat. Initially, the flow is set sub-optimally (i.e. about 1ml/hr) to avoid over-dosing from the remaining drug intraperitoneally. After an hour, the max flow is set to render the full calculated dose of the drugs.

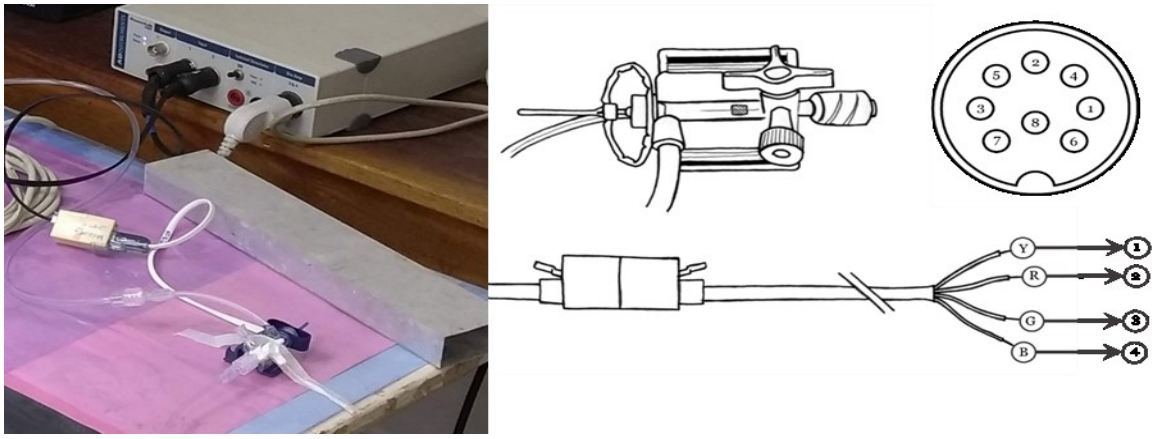


***Figure 13*: Connection for the iPeX transducer to make it compatible with the AD instrument data acquisition systems.**

iii. *Other considerations before neck dissection*: The respiratory belt is tied around the anesthetized Wistar rat at the thoracic and abdominal junction. Disposable adhesive button ECG electrode was placed on the right and left upper limb, and the left lower limb. The limbs were secured to the flat surface, and ECG leads were connected, POSITIVE – to the left (brown), NEGATIVE to the right (white), and GROUND to the left leg (green) button electrode (figure 17). The UPS and computer are turned on, and the LabChart

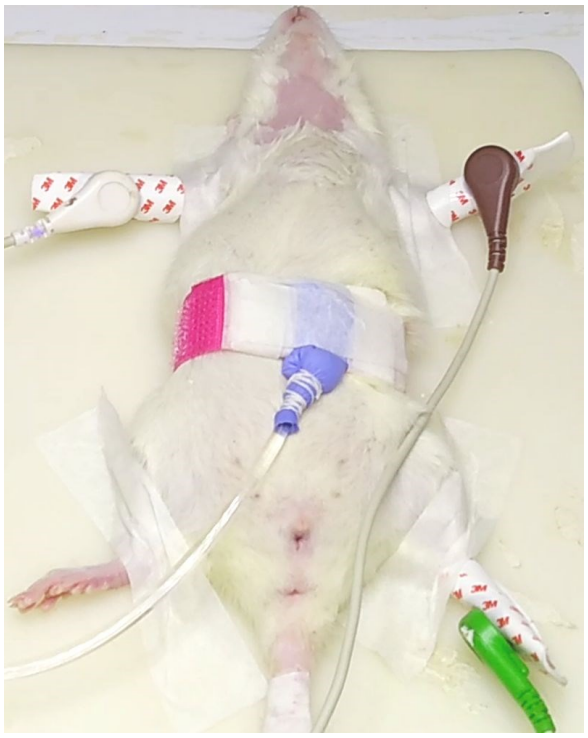


***Figure 14*: ECG electrodes are applied to the two upper limbs and left lower limb. Green – ground cable, Brown – positive lead to the left arm, White – the negative lead on the right arm.**

software data acquisition software is run after connecting the ADinstruments via USB cable.

iv. *Calibration and set-up of the transducer*: The iPex pressure transducer (figure 18) measures the changes in the pressure in a fluid-filled system. The parts of the transducer and its various connections are shown in the figure below. The iPex pressure transducer was not compatible with the AD instrument device, and so a custom-made connector pin had to be made (see FIGURE 16). This was done in a separate study, by a post-graduate in

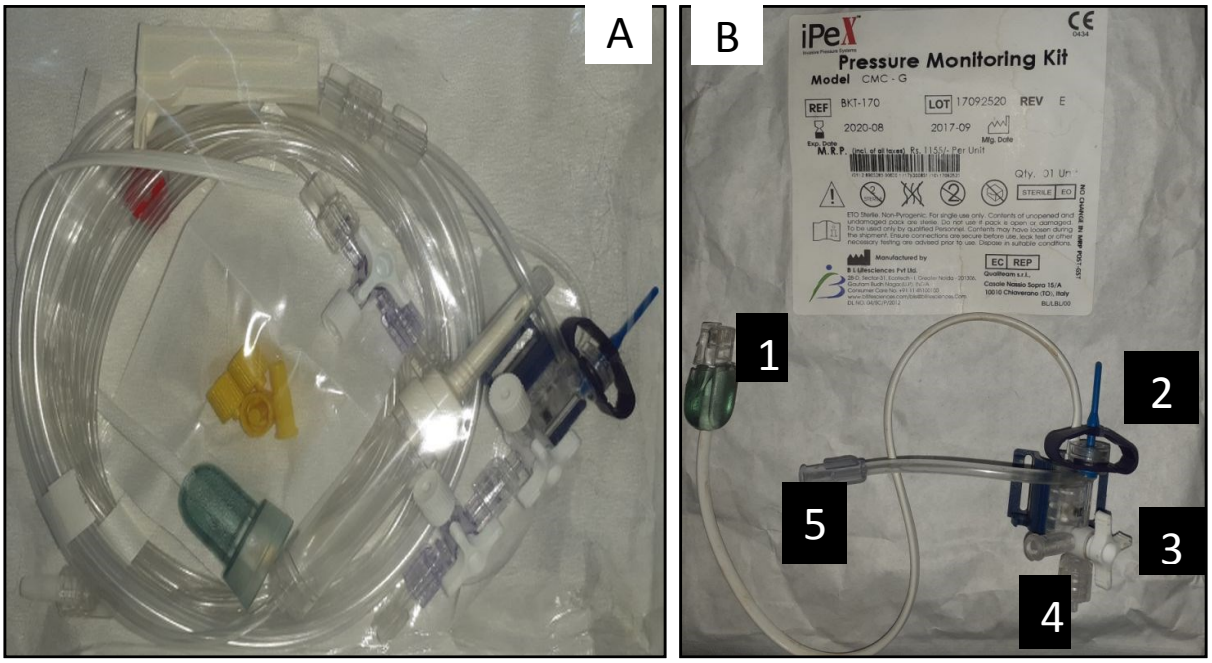


***Figure 15*: The iPeX® pressure monitoring kit.**

A. The Pressure monitoring kit with the extensive tubing and connector system used for intra-arterial pressure recording.

B. The transducer; 1) Electrical output, 2) rubber teat to perform flush test, 3) 3-way knob, 4) Connection for the tubing IV catheter, 5) connection to pressurized saline.

the same department. The following figure is adapted from that study, which helped in making the right connections. A similar connector was made for the respiratory belt. When the pressure transducer was connected to the AD instrument via the connector, pressure tracing was obtained, but now there was this need to calibrate it. A pressurized bad with 500mL normal saline in it is connected to the pressure transducer as shown in figure 31. The pressure was raised to 300 mmHg. For the experiments, 20 µL of heparin (100 IU) was added to the saline for preventing disseminated intravascular coagulation. Calibration is the process of defining the relationship between a unit change in the electrical signal (mV), which is the output from the transducer in response to the changes in the fluid movement inside the vessel, and the change in the pressure value in an aneroid barometer. This is quickly done in the LabChart software 2-point unit conversion. The aneroid barometer is

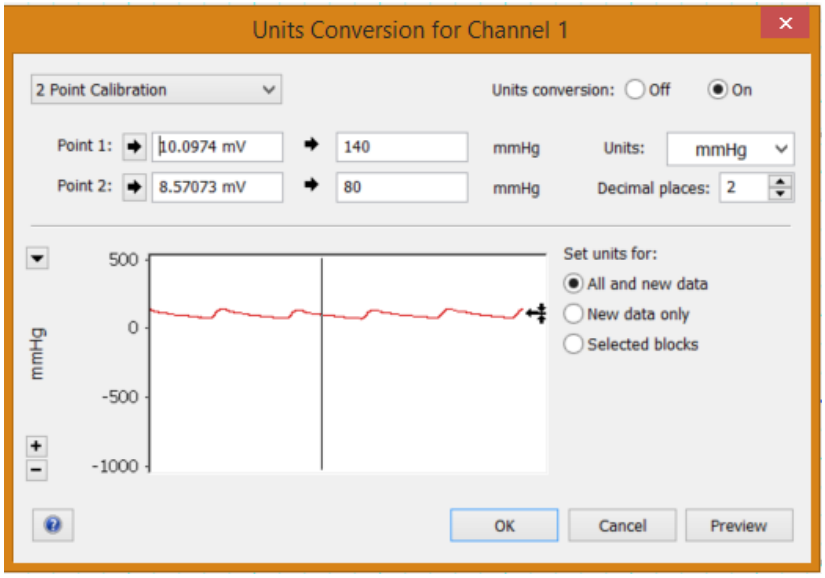


***Figure 16*: Snapshot of the dialog box that enables to calibrate in ADInstruments.**

connected to the pressure transducer using silicone non-compliant tubing along with a 3-way connector in the circuit. First, the 3-way is turned toward the external atmosphere and then turned to make it a closed circuit such that when the bulb is pressed, there is a deflection in the pressure channel tracing. Now elevate the pressure inside to two values and hold it for about 10 seconds and then let go the pressure. Now the pressure tracing that was recorded had two distinct levels. Place the cursor on the first level and feed the corresponding pressure value against it. Do this for the second pressure level. Change the units to mmHg. A sample is shown in figure 19. Mark the corresponding voltage values with those two points against their mV values. Press OK. Then manually elevate the pressure in the barometer and see whether the values read by the software are the same as that in the aneroid barometer. An error of ± 5 mmHg is considered acceptable. If not, then repeat the calibration procedure. Changing the pressure transducer was practiced after every five experiments and calibration was done prior to every experiment. It was also common practice to perform the fast-flush test for an intra-experimental check on the pressure measurement.

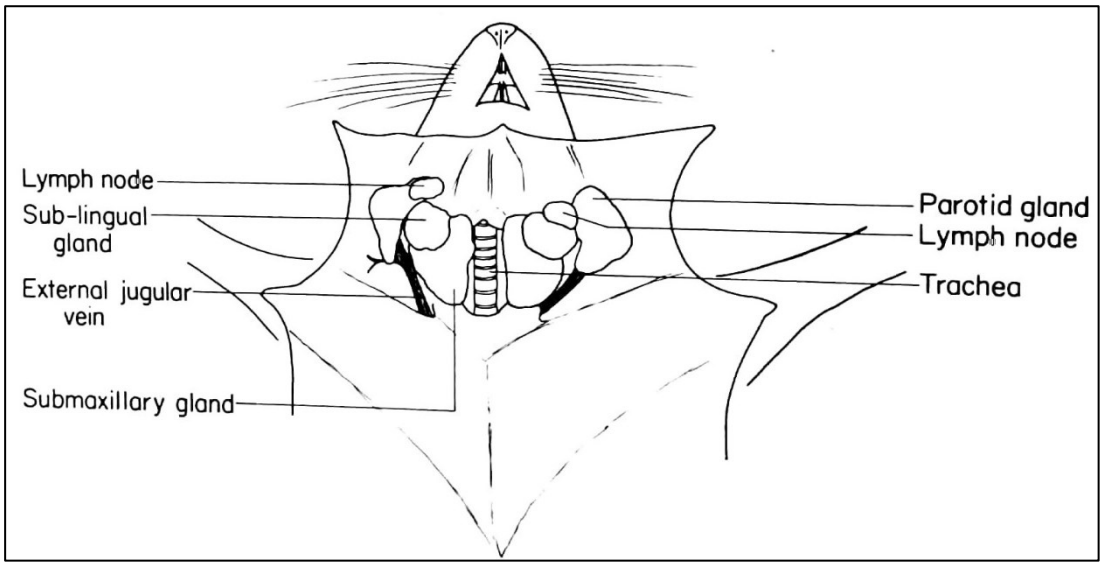


***Figure 17*: A schematic diagram of the main structures that are encountered after removing the skin flap.**

v. *Anatomy of the carotid artery in the neck*:

The know-how of the anatomy of the neck helps in dissecting the structures carefully, avoiding damage to essential structures (figure 20). The layers of

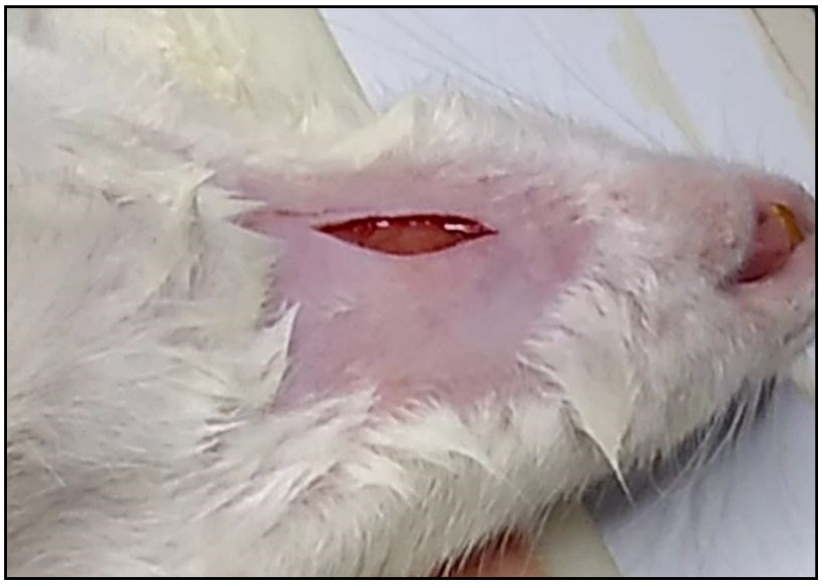


***Figure 18*: The hair over the neck region is shaved and a straight incision is made from the jaw to the thorax parallel to the midline.**

anatomical structure encountered during the surgery to dissect out the common carotid artery (CCA) are, from anterior to posterior:

A. The skin
B. The salivary glands
C. The muscular layer – consists of many paired muscles, namely the sternomastoid muscles, sternohyoid, omohyoid. Digastric Ms. is found in the upper part.
D. The fascial plane between the different muscles and the glands.
E. The carotid sheath, which is an investment of fascia around the CCA.

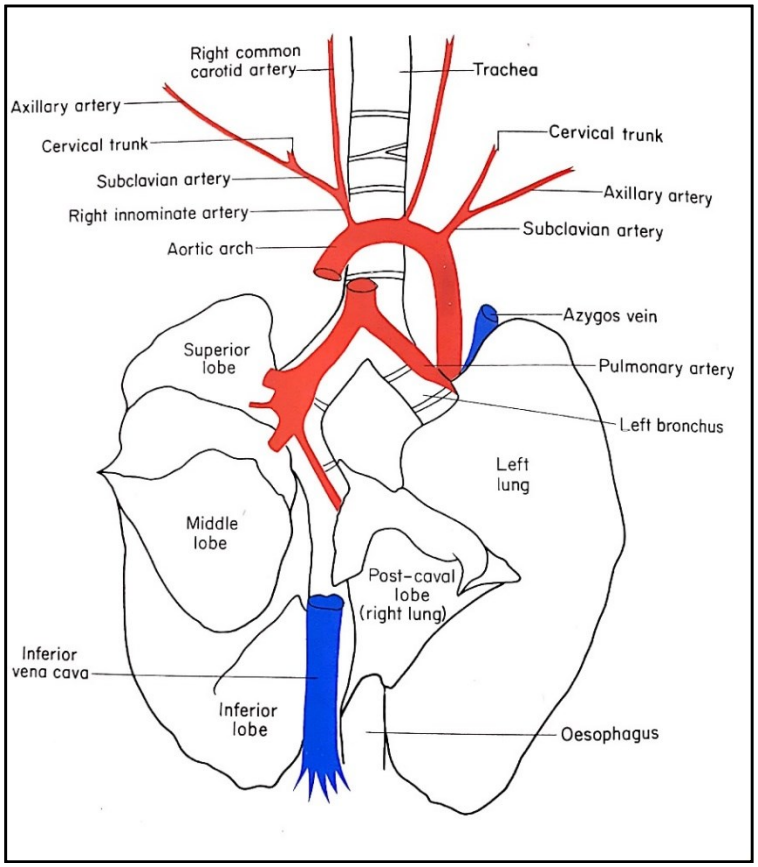


***Figure 19*: the above diagram shows the major artery systems in the neck region and upper thorax.**

F. The sympathetic trunk and the Vagus nerve which can be easily identified because of its thick white appearance and is rarely missed. The sympathetic trunk, however, is hard to see and is easily damage while dissecting out the carotid.

G. The carotid artery which lies parallel and behind to the trachea on both side (figure 22).

vi. *Dissection to reveal the carotid artery*: The entire neck and upper thoracic areas are shaved and first wiped with 70% ethanol. Using no.11 scalpel blade, an incision is made from the mandibular region 2-2.5 cm to the manubrium sterni just parallel to the midline. The incision can be extended towards the scapulohumeral junction if needed (figure 21).

The superficial veins cut in the process of extending the depth of the incision results in bleeding that is manageable. Using a curved forceps, the fascia is

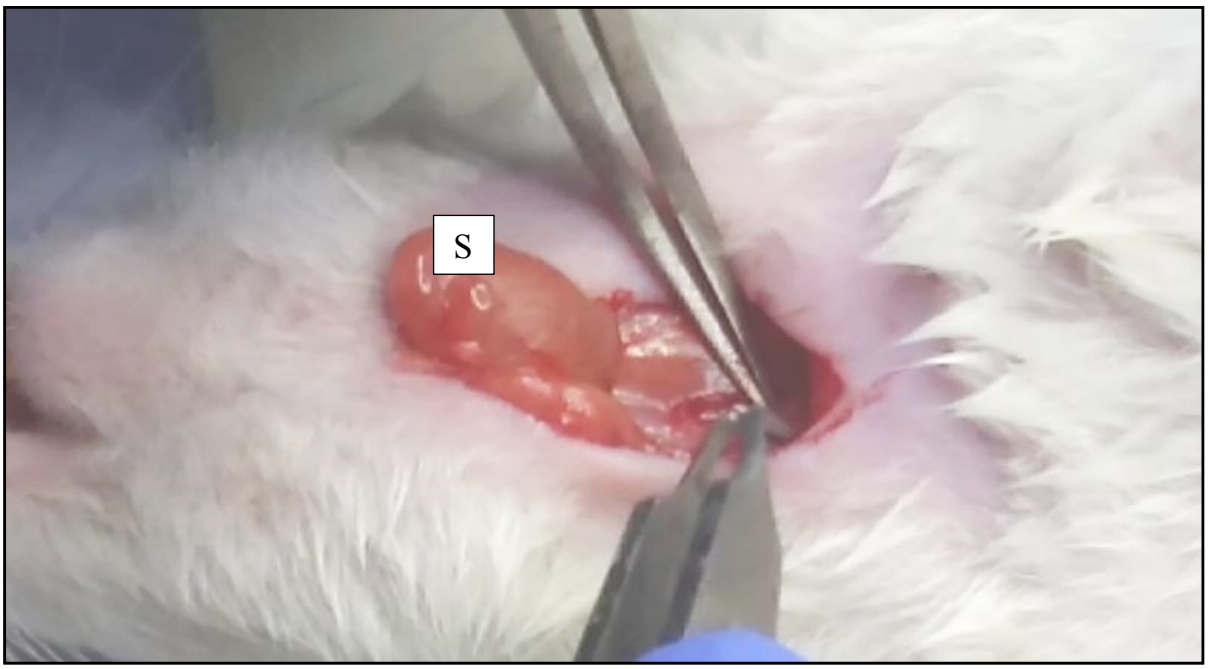


***Figure 20*: S, submaxillary gland, is shown here pulled up and flapped over the skin to clear away a major structure in the neck.**

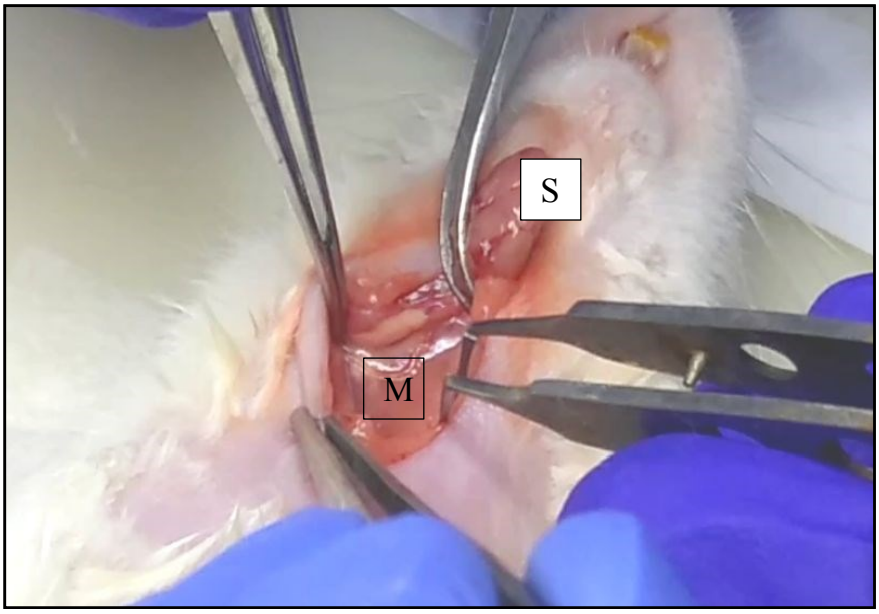


***Figure 22*: S, submaxillary gland, M, muscular area i.e. one can see all the anterior neck muscles in and around trachea.**

just pulled apart exposing a neat muscular plane underneath in the middle part

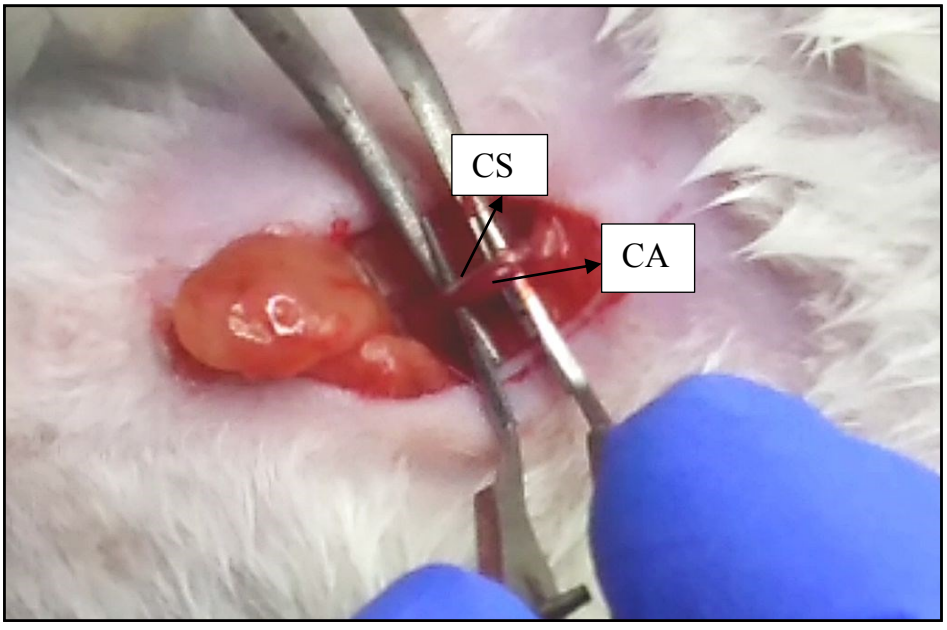


***Figure 21*: CS, Carotid sheath; CA, Carotid artery. The carotid artery along with the sheath is dissected out from its attachments to the Vagus nerve and sympathetic nerves.**

of the incision, and the salivary glands are seen in the superior part of the incision. Now using the forceps clear off the fascia and through the avascular area expose the salivary (submandibular and sublingual) glands. This is a rather large structure that can be easily lifted and reflected on the skin, as shown in the figure (figure 23, 24 & 25). Once the muscles and the trachea are seen, the middle part of the field is selected for further dissection. This is the field where the least number of structures overlap the carotid artery (60). The muscles are seldom cut, but sometimes, the omohyoid is cut to clear the area. The sternohyoid muscles must be cut to reach the artery. The Vagus nerve is a solid white-colored structure that is easily identified. The artery is always close to it. Another way to identify the artery is to look for the definite pulsations. Through the fascia around the carotid sheath (figure 24), lift the neurovascular bundle and pass a right-angled or curved forceps underneath it.

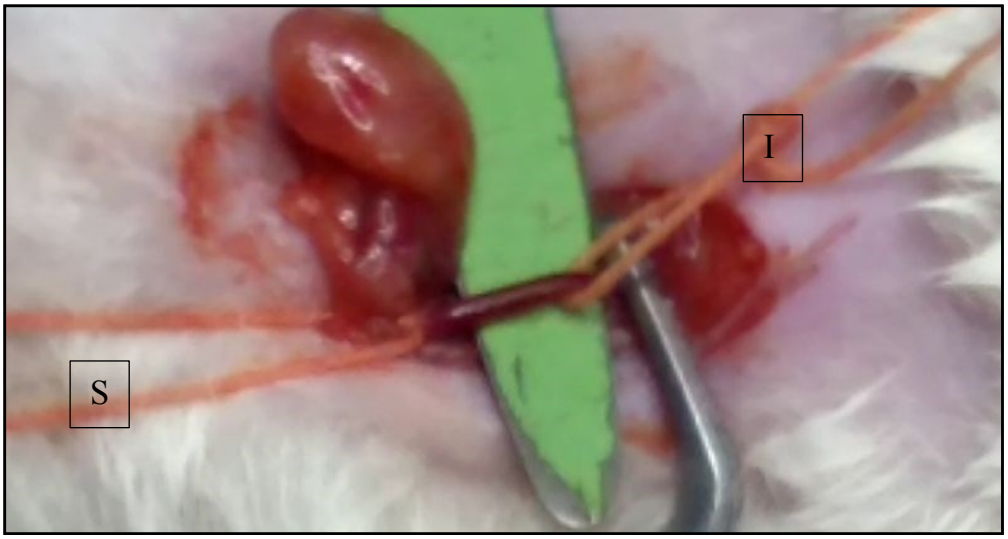


***Figure 23*: The Spatula and the bulldog clamp is attached. The superior thread (S) is tied as high as possible and the inferior thread (I) is left untied. The spatula keeps the cannulation stable.**

This frees the bundle from the posterior wall. Now start separating the various structures one by one until the carotid artery is made available alone. We prefer keeping the sheath intact as it gives more stability for cannulation. However, some prefer to remove the artery from its sheath. In our experience, we have found that bleeding from the sides is maximal when the sheath is removed while trying to push the cannula further in once the bulldog clamp is removed.

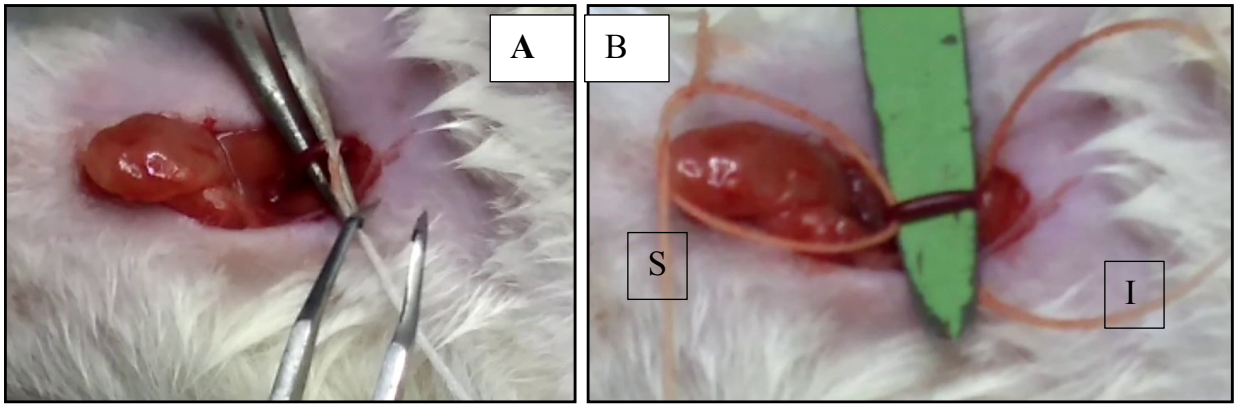


***Figure 24*: A. Thread being passed under the carotid bereft of its sheath. Two such threads are looped around the artery as shown in B. S – superior thread i.e. towards head end. I – inferior thread is near to the heart**

Once this middle part of the vessel is free of all surrounding structures like nerves and other vessels, stretch the artery gently from superior to inferior direction along the path of the vessel underneath it. This gives a good portion of the artery free. Pass two threads soaked in NS under the artery. Place one inferiorly and the other superiorly. Prepare a loop with the inferior thread and place it without entirely tying a knot. With the superior thread, tie a knot as high on the artery as possible. Pass a spatula under the artery, and then clamp

the carotid artery using a bulldog clamp as close to the heart as possible (See figure 26, 27, 28).

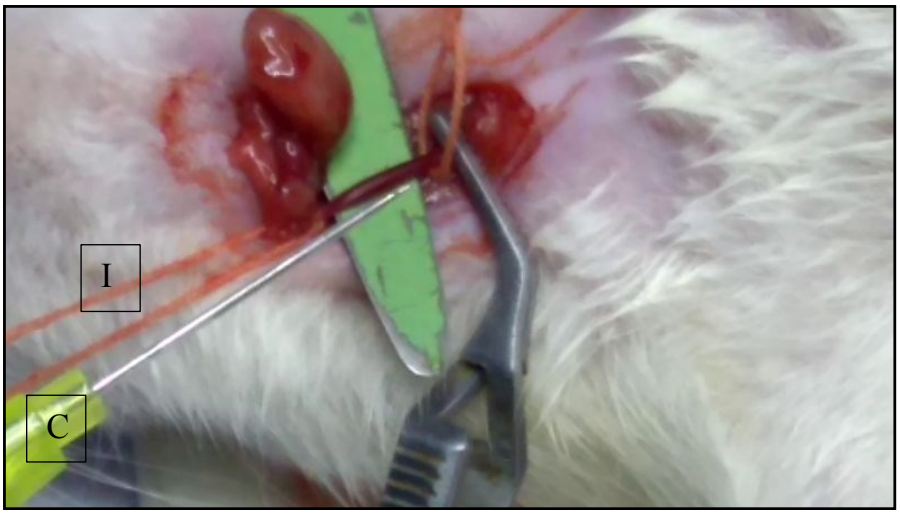


***Figure 25*: The inferior thread is pulled And the Hep-lock flushed 24G cannula (C) is kept ready.**

Now a 24G cannula that is already kept ready flushed with hep-lock solution is taken, and its protective plastic is removed. Position the cannula properly and cannulate the artery towards the direction of the heart while continuously giving gentle traction on the artery superiorly using the knotted thread. If the

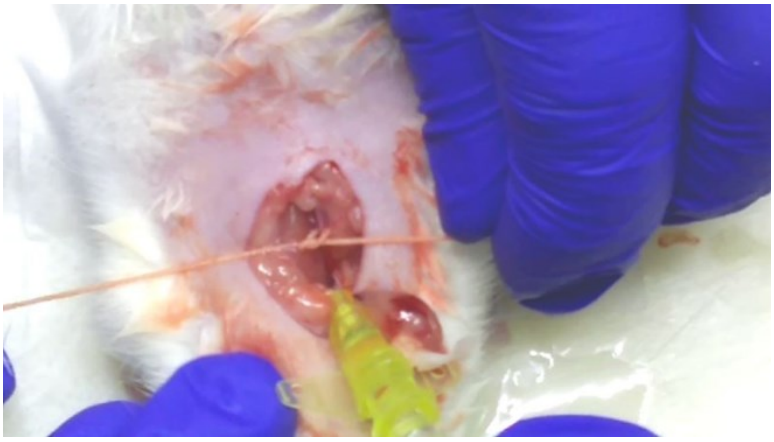


***Figure 26*; The Inferior thread is used to tie the cannula in place once inside the lumen of the artery preventing it from slipping out. Tie both the thread to the cannula.**

cannula is in, push the cannula further in, while at the same time withdrawing the needle from it. Now flush the catheter with the hep-lock solution using the same needle till the end. Use the heparinized-saline-filled tube that is connected to the calibrated iPex pressure transducer (as mentioned above), to connect to the carotid artery catheter. Ensure that the whole catheter tubing system is free of all possible air trapping. Now remove the bulldog clamp; there should be no leak of blood. Push the catheter as far into the lumen as possible and tie a secure knot using the inferior looped thread, which maintains the catheter in place without slipping off (figure 29, 30). Tie the superior thread also to the catheter for added security. Cut the excess ends of the thread. If correctly in the lumen, one can see the pulsations of the artery visibly inside the catheter lumen and pressure recordings in the software

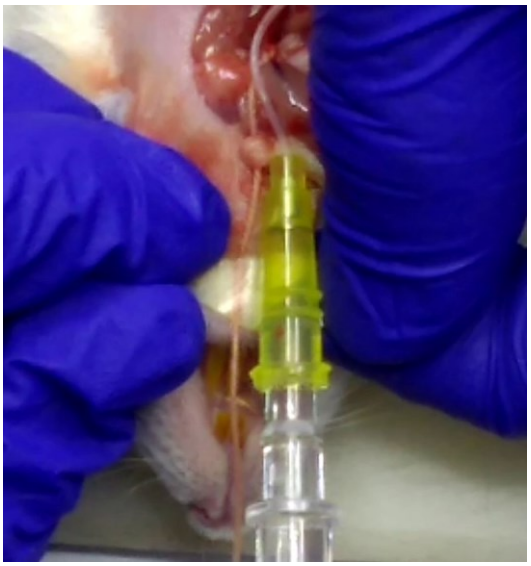


***Figure 27*: Once the yellow catheter is in the lumen the catheter is filled to the brim with hep-lock solution. Then the end of the transducer tubing system, filled with heparinized saline, is connected to the IV cannula. Remove any air bubbles before this step from the catheter.**

should now be the tracings of the typical arterial pressure waves (figure 31). Connect the oxygen mask to the nose and start giving a small dose of oxygen.

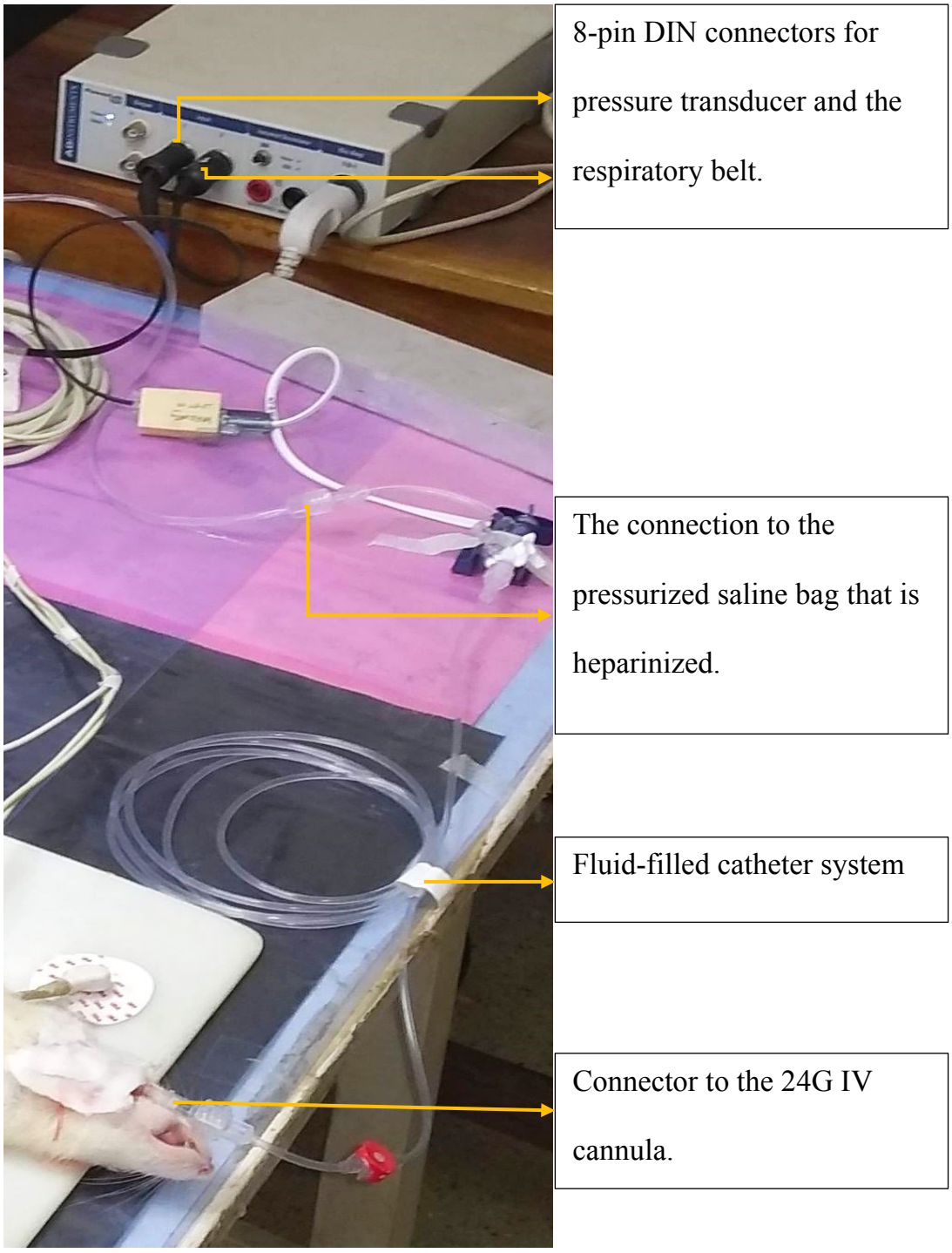


***Figure 28*: The whole pressure recording line – from the catheter inside carotid artery to the ADInstruments DAQ.**

5.1.F) Animal carcass disposal

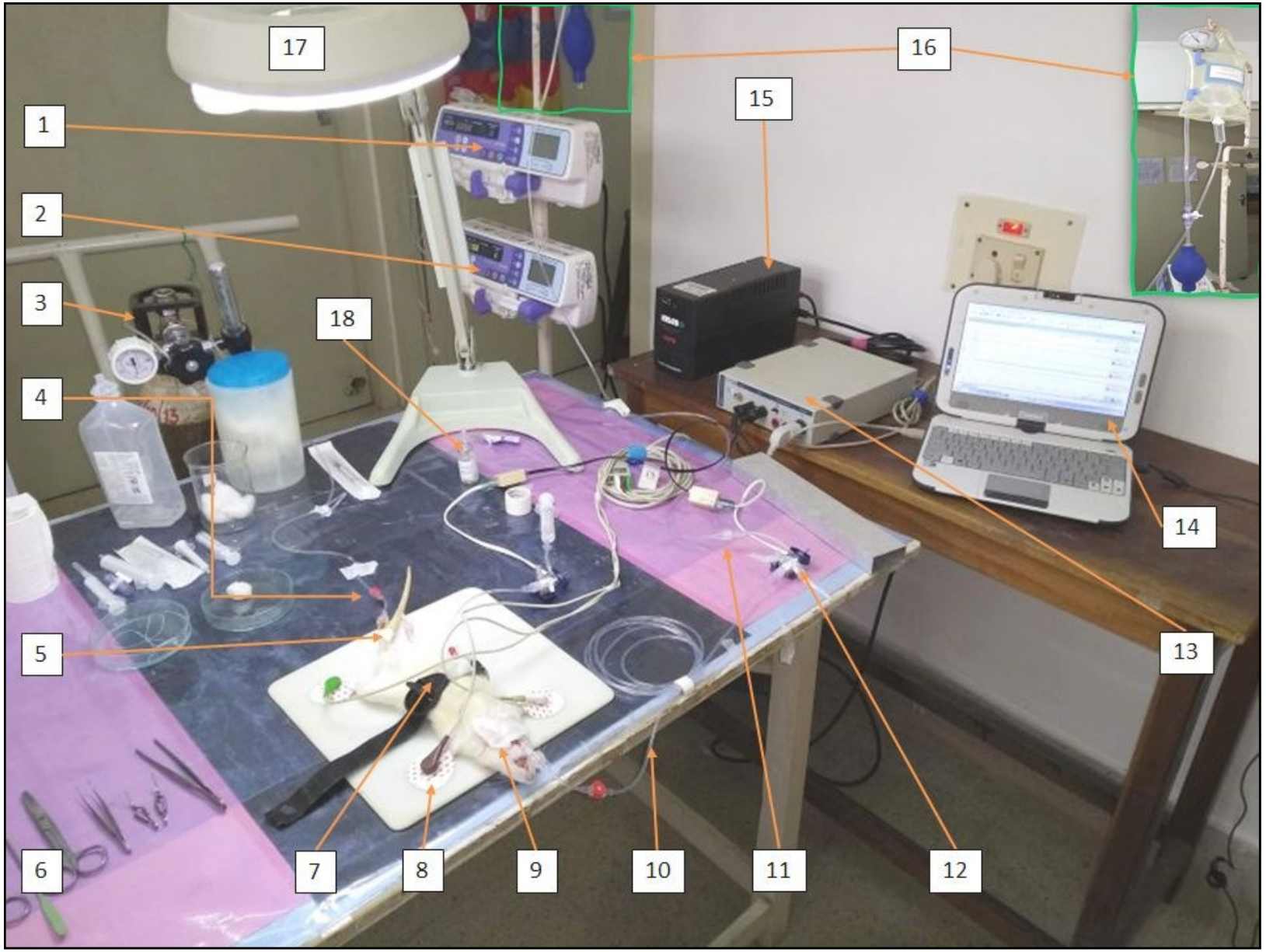
The animals that were either sacrificed at the end of 8 hours by an overdose of anesthetic or those that die due to septic shock because of the organism were all disposed of according to the bio-safety standards. The place was wiped clean, with 70% ethanol.

5.2. Experiment Schema

5.2.A) Complete Experiment Schema

The complete experimental setup looks like the figure shown below (figure 32; see next page):

Figure 29*:* The whole setup of carotid artery pressure recording from Wistar rats. 1) Infusion pump for drug, 2) Infusion pump for maintenance anesthesia, 3) Oxygen cylinder, 4) an aesthetic line connected to the tail vein catheter, 5) Lateral tail vein cannulated, 6) Surgical instruments, 7) Respiratory belt connected to its transducer, 8) ECG leads connected to the limb, 9) Carotid artery cannulated and covered with a Saline soaked gauze, 10) Fluid-filled catheter tubing connected to the iPeX transducer, 11) Pressurized bag connected to transducer, 12) iPeX pressure transducer, 13) ADInstruments data acquisition device, 14) Laptop with LabChart pro software, 15) UPS, 16) Pressure bag also shown in inset picture, 17) Light source with magnification lens for microdissection, 18) Hep-lock flush solution.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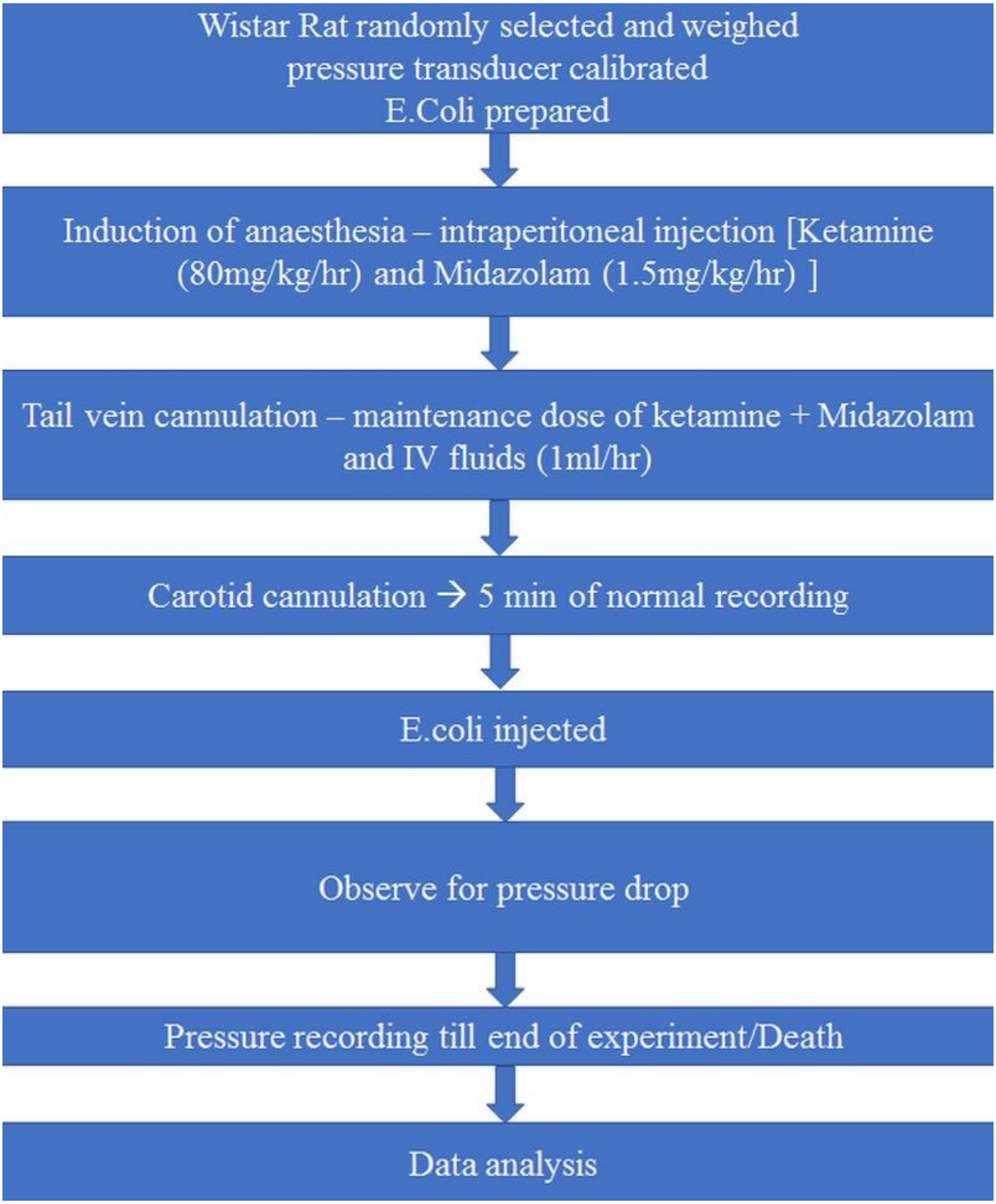
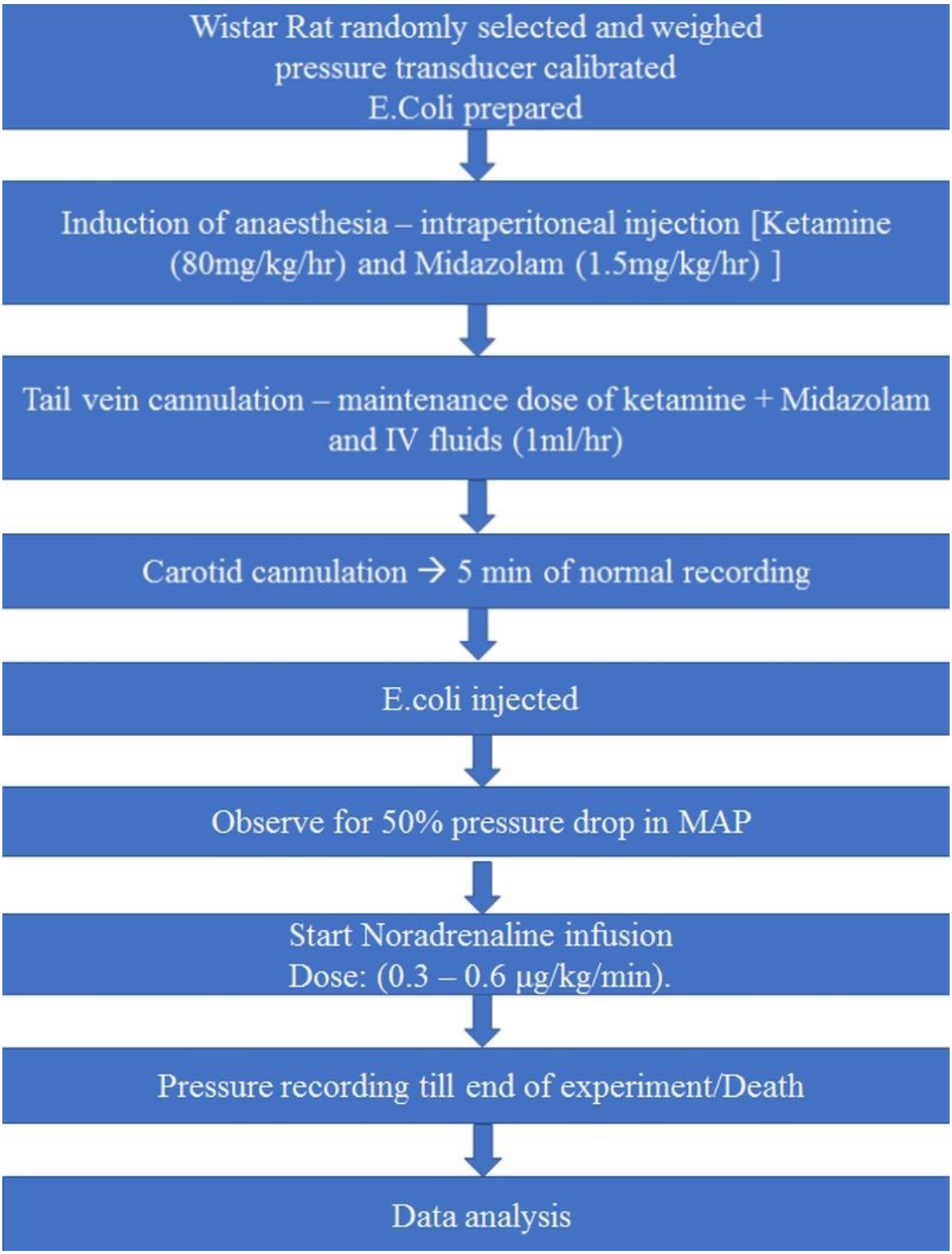
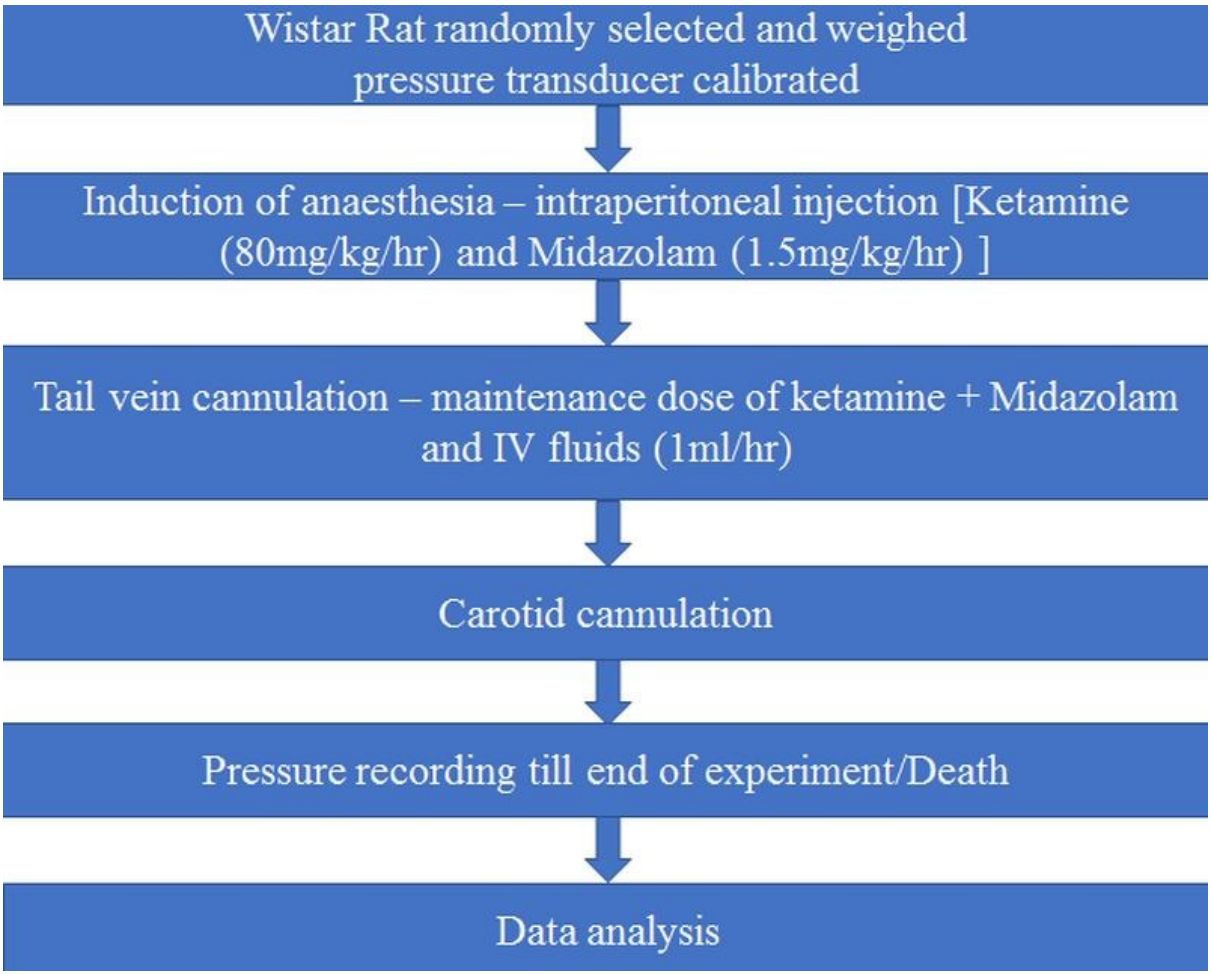
5.2.B) The **Test arm schema** is given below:



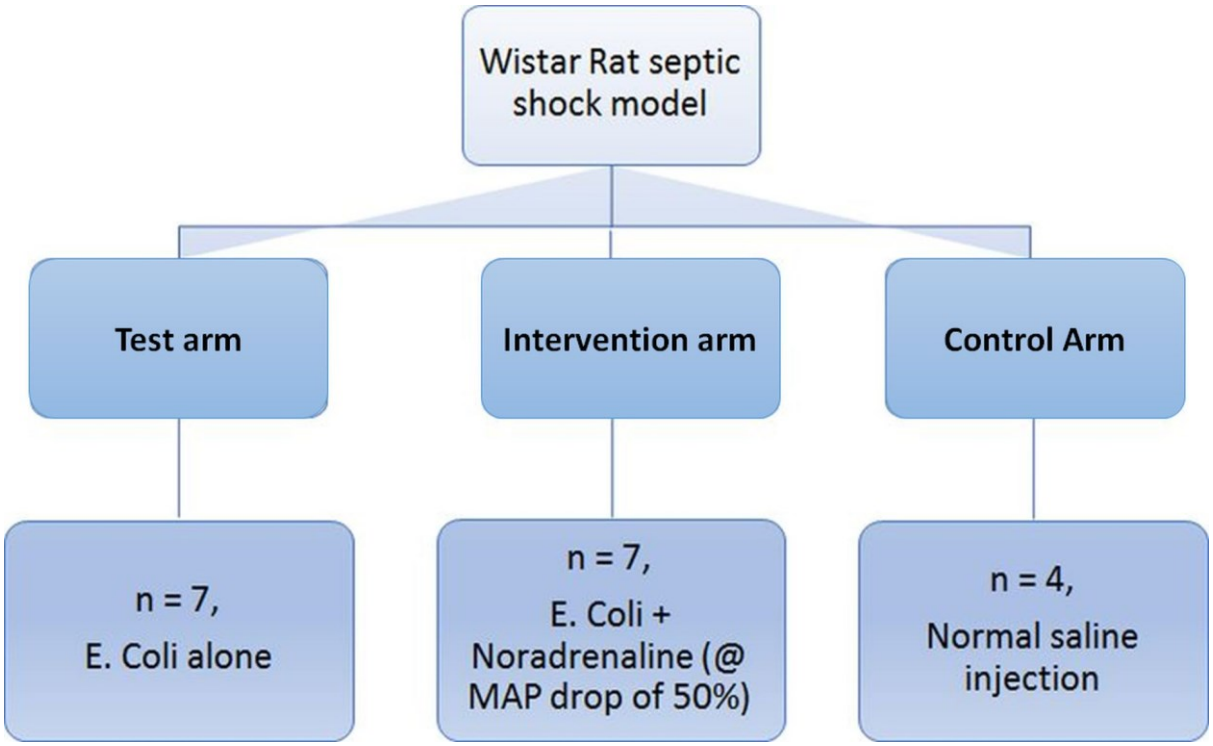
5.2.C) The **Intervention arm schema** is as shown below:



5.2.D) The **control arm schema** is given below:



5.3. The whole experiment schema is given below:



CHAPTER 6 ANALYSIS:

6.1. Preliminary considerations:

The pressure recordings obtained from an experiment were of an average duration of 6-7 hours. Such extensive data need better equipment. Computers have become faster and can process data quicker, providing us with capabilities that can help us handle massive data sets. Most commonly used languages used in data analytics are Python, R, SQL, MATLAB®, IGOR.

Data analysis for this experiment was done using a custom-made program in Matlab®, a high-performance language used by many scientists and engineers for technical computing. The recording was done on "ADINSTRUMENT PowerLab 15T®" with two analog input connectors and a Bio Amp connector for ECG recording (figure 33). The software comes with this instrument called LabChart®. The whole experiment was recorded in this and stored in the '`.adicht`' format. Later, it is exported to the '`.mat`' format for use in Matlab.

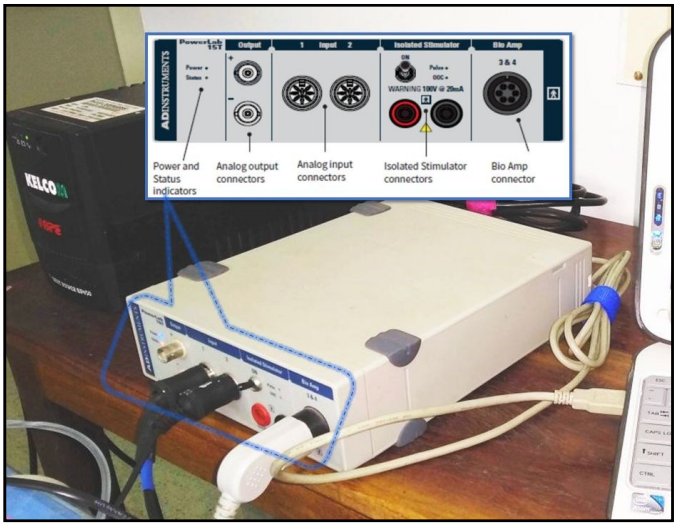


***Figure 30*: ADInstruments device with inset showing different connection.**

6.2. Parameters to study:

The following parameters were studied -

a. The systolic and diastolic pressure
b. The mean arterial pressure (MAP)
c. The heart rate.
d. The pulse pressure.
e. These above-mentioned physiological parameters were obtained using the program from the following instances:
    i. Before the addition of *E. coli*.
    ii. Before death of the animal.
    iii. Just before the addition of the intervening drug, in this study, noradrenaline.
f. Measurements of different time intervals: In the first group (*E. coli* alone), the time it took (t_1) for the MAP to drop 50% from the MAP observed at the beginning of the experiment was measured. Also measured was the time it took for the animal to die from the moment of 50% MAP drop (t_2). Similarly, in the intervention group (*E. coli* + Noradrenaline) the time it took to observe the fall in MAP (t_1) and the survival time from the drop in MAP (t_2) were calculated by running different scripts of the program. 't2' in this group is the expression of the effect of Noradrenaline.

6.3. The logic behind the code:

Any code written is as good as the logic behind it and the algorithm written to find the solution to the task at hand. All the code written for this experiment is given in APPENDIX. The primary algorithm used is as following:

a. Load the .mat file

b. Make the selection from the data

c. Find the peaks and troughs for those selections.

d. Using the peaks and trough values and their location one can find the mean of the systolic, diastolic and the running mean of the individual selected sections

e. Plot the same on the sections of the graph and save the variables.

f. For depicting the whole experiment, again, load the whole file.

g. Find the peaks and troughs of the entire data. Find the running mean of each wave and plot the mean on a graph.

h. Plot the location of the various events using their location points on the graph.

i. Calculate the time intervals

j. Save the values and print the data.

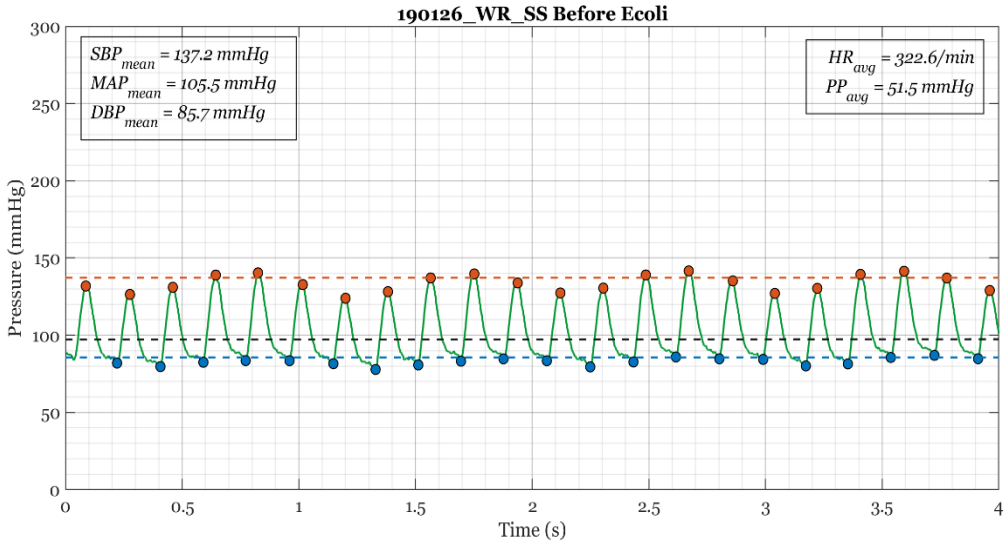


***Figure 32*: shows a small section of the one-minute data selected before the addition of E.coli, from the complete data of a single experiment. The values of the systolic (SBP_{mean}), diastolic (DBP_{mean}), mean arterial pressure (MAP_{mean}), heart rate (HR_{avg}), pulse pressure (PP_{avg}). This is representative**

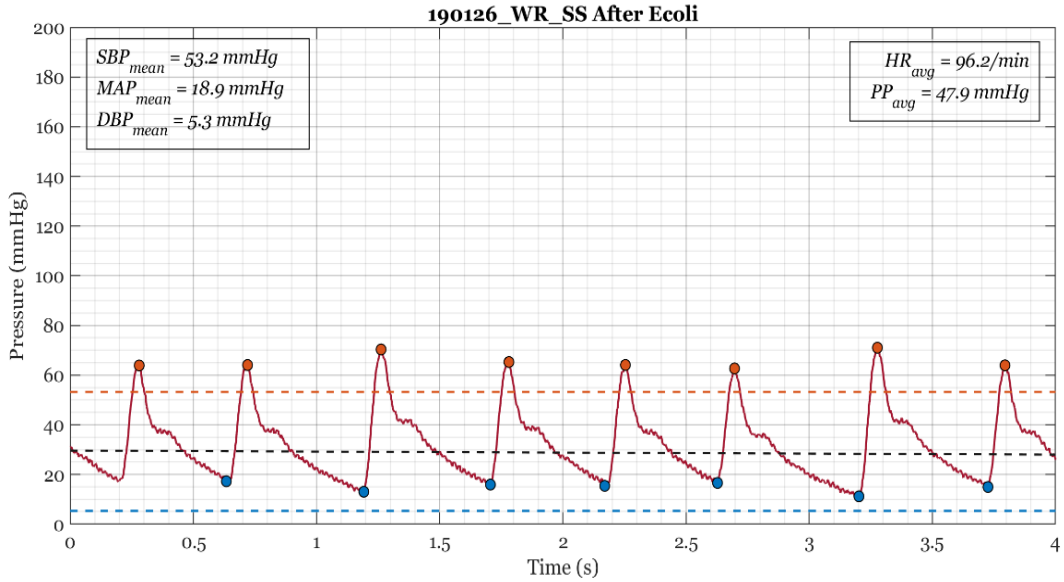


***Figure 31*: shows a small section of the one-minute data selected after the addition of *E.coli* just before death. The values of the systolic (SBP_{mean}), diastolic (DBP_{mean}), mean arterial pressure (MAP_{mean}), heart rate (HR_{avg}), pulse pressure (PP_{avg}) are given. This is representative data from Group 1 (*E.coli* only).**

6.4. Analysis of the different groups:

6.4.A) Group 1 – test arm:

In this arm of the study, E. coli was added following which the pressure dropped, and the animal died. Two sections were selected, of one-minute duration, from the whole experiment; one before the addition of *E. coli* and one just before death. The parameters (a-d), mentioned above, from the code in both these one-minute-long sections of data. In the next code, the whole experiment is plotted and, the following three locations are plotted (figure 34, 35, 36); First, the instance when *E. coli* is added, second, the point at which a 50% drop in MAP was noted and third, the location on the graph when the

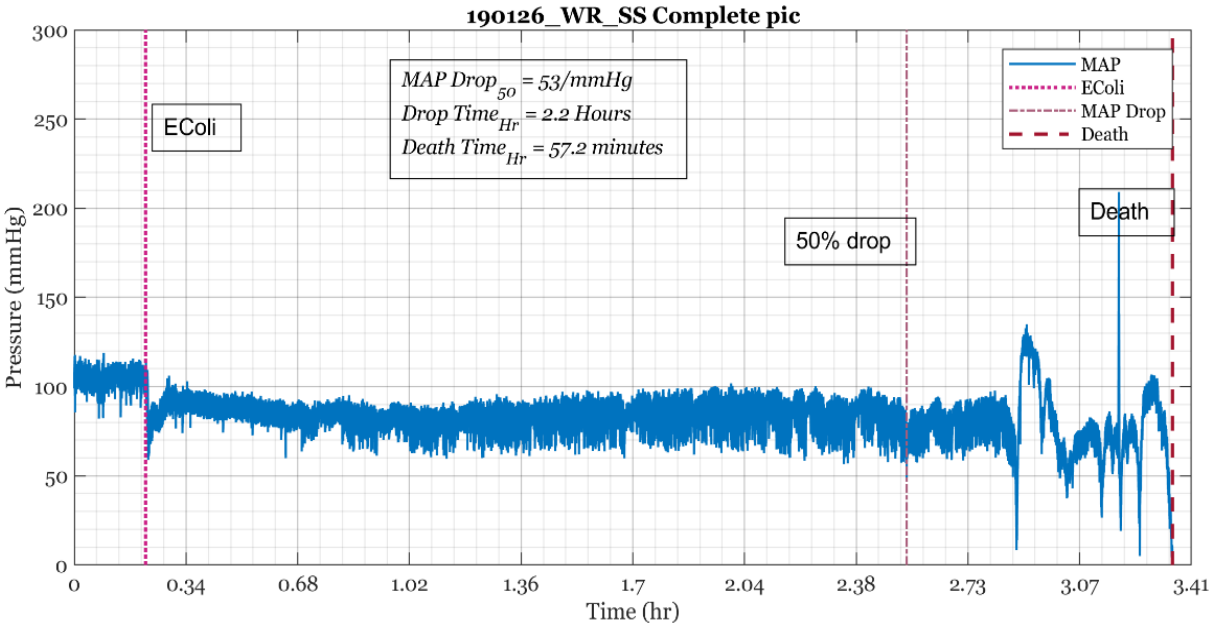


***Figure 33*: Complete experiment plot. The values of the drop in MAP i.e. 50% of initial mean (MAPDrop50), time interval between addition of EColi to 50% MAP drop (Drop TimeHr) and the time taken to die from the point of drop (Drop TimeHr). This is representative data from Group 1 (E.coli only).**

animal died. T1 is the time interval between the 1st and 2nd locations and, T2 is time interval between locations 2nd and 3rd, as shown in FIGURE 36.

6.4.B) Group 2 – intervention group:

This group consisted of rats were both *E. coli* and Noradrenaline after a drop in MAP were administered. Here we evaluated three sections – before *E. coli*, before the intervention, after the intervention (i.e. before death or end of experiment). Data from these three sections parameters (a-d) were run through the code which generated values for each parameter. Another script of code processed the whole file and plotted the complete experiment. On this, Matlab-generated plots, the instances of *E. coli* injection, 50% drop in MAP and the time of death. We got the time durations between the three events just like above (See figures 37, 38, 39 & 40).

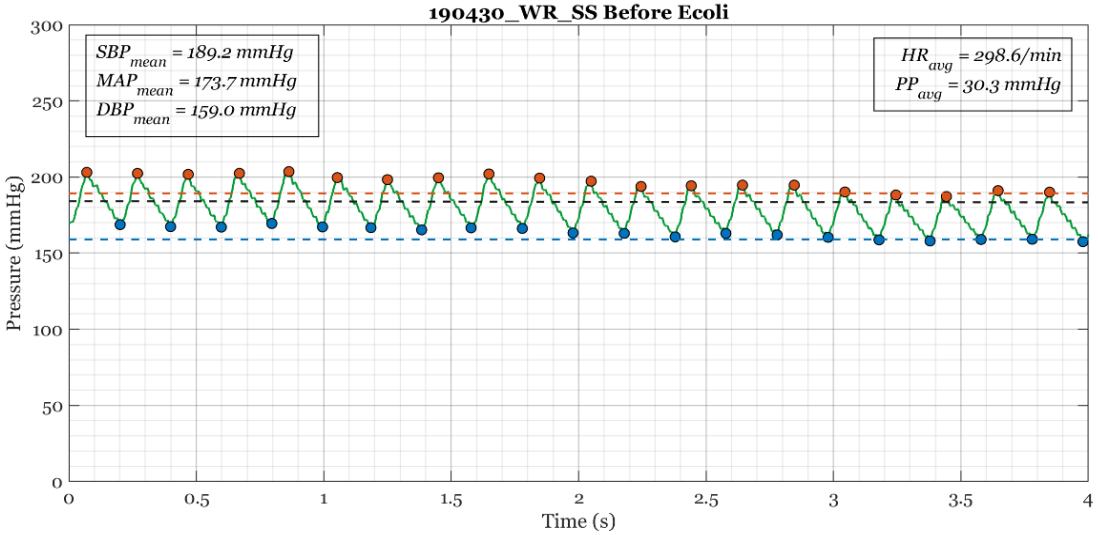


***Figure 34*: one-minute section data before the addition of E.coli. The values of the systolic (SBPmean), diastolic (DBPmean), mean arterial pressure (MAPmean), heart rate (HRavg), pulse pressure (PPavg). This is representative data from Group 2 (E.coli + Noradrenaline).**

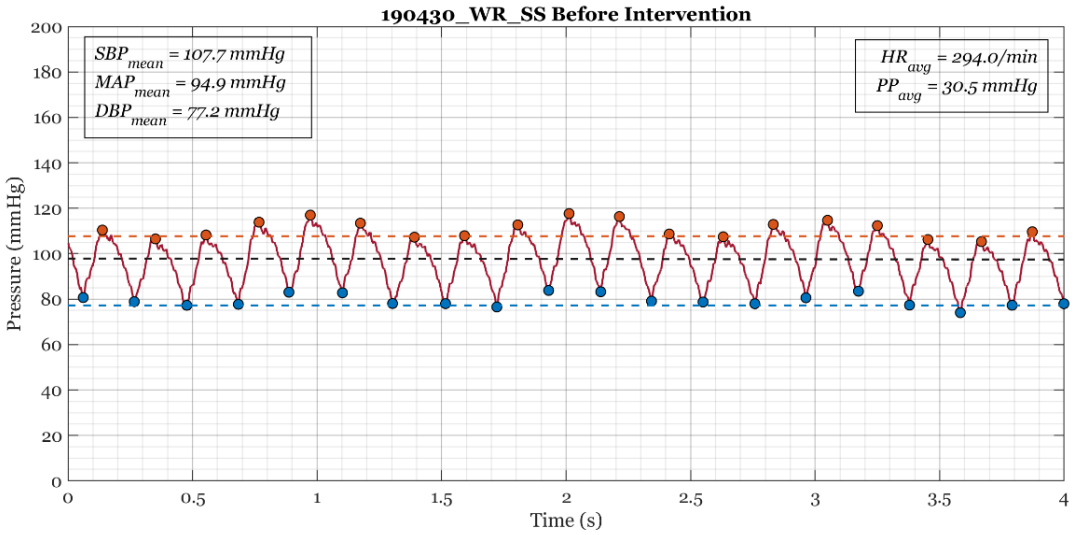


***Figure 36*: one-minute section data after pressure drop before the addition of Noradrenaline. This is representative data from Group 2 (E.coli + Noradr).**

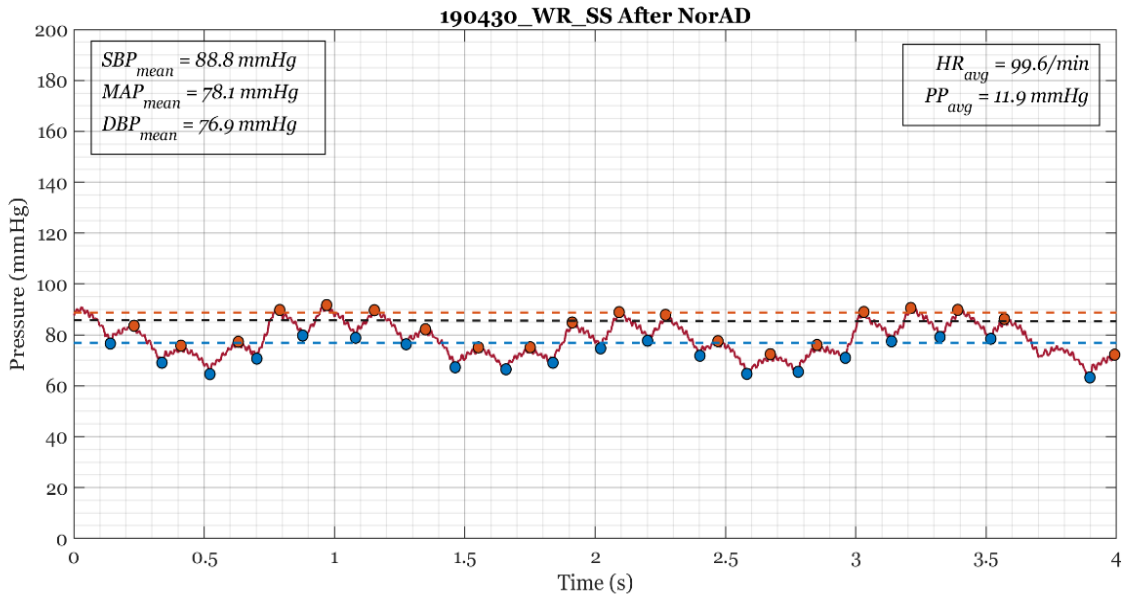


***Figure 35*: Effect of addition of Noradrenaline. The values of the systolic (SBPmean), diastolic (DBPmean), mean arterial pressure (MAPmean), heart rate (HRavg), pulse pressure (PPavg). This is representative data from Group 2 (E.coli + Noradrenaline).**

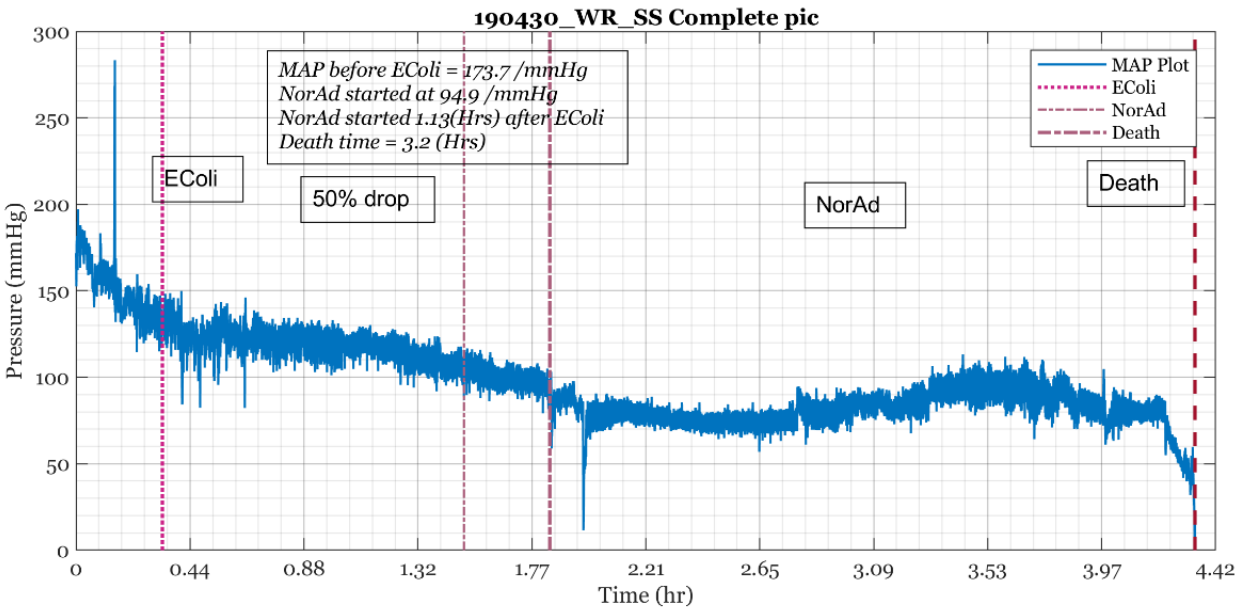


***Figure 37*: Complete experiment plot. The values of the drop in MAP, time interval between addition of EColi to 50% MAP drop (Drop TimeHr) and the time taken to die from the point of drop (Drop TimeHr) are calculated and noted. This is representative data from Group 1 (E.coli only).**

6.4.C) Group 3 – (control arm)

Here, Normal-Saline solution is injected alone. The average duration of these experiments was about 8 hours. Animals who survived for about 8 hours or more were euthanized with an overdose of the anesthetic cocktail.

CHAPTER 7 RESULTS

7.1. Septic shock

The primary goal of our experiment was to create an animal model of septic shock using live strains of *E. coli* bacilli. Furthermore, for that, we injected them intravenously and observed a reduction in blood pressure, and subsequently the animal died of severe hypotension.

***Table 6*: This is from Group 1 and Group 2, n=14. The effect of *E. coli* on blood pressure. The time taken for 50% drop in MAP is shown here.**

Descriptive statistics		The time it took for MAP drop (in Hrs)
Total no. (n)		14
Mean		1.94
Median		1.54
Std. Deviation		0.97
Minimum		0.60
Maximum		4.22
Percentiles	25^{th}	1.33
	50^{th}	1.54
	75^{th}	2.54

A decline in the MAP of 50% or more was chosen to indicate onset of shock due to sepsis. In groups 1 and 2, when *E. coli* was given, the time taken for the septic shock to develop was found to be as shown below in the table 6. The average time for the *E. coli* dose to cause hemodynamic shock was close to two hours (1.94 ± 0.97 hours). The maximum time taken was about 4hrs and minimum time 0.6hrs.

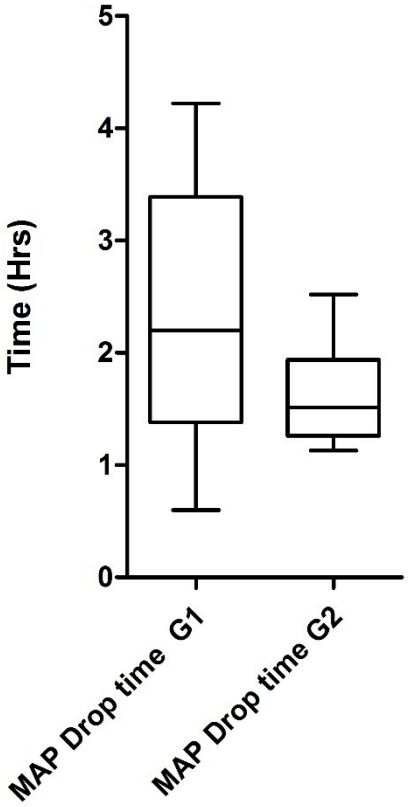


***Figure 38*: This is a plot of the time taken for MAP to drop in both the groups (G1 – group 1, G2 – Group 2).**

This shows the biological variability or the difference in the susceptibility to *E. coli* within the same species (figure 41). We can also notice that 75% of

the animals had septic shock within two and a half hours after *E. coli* administration.

7.2. Survival time after the septic shock:

In group 1 (n=7), where only live bacteria were injected, once the animal had developed shock, i.e. 50% drop in MAP, we studied the distribution of the time of survival after the onset of shock. The values are given in TABLE 7:

***Table 7*: This is from Group 1, n =7. The time taken for death to occur after 50% drop in MAP is evaluated here.**

Descriptive statistics		**Time to MAP drop (in minutes)**	**Time to death (in Minutes)**
Total no. (n)		7	7
Mean		136.43	38.2422
Median		131.94	32.39
Std. Deviation		74.77	20.56
Minimum		36.00	9.90
Maximum		253.20	62.30
	25th	82.75	21.06
Percentiles	**50th**	131.94	32.3866
	75th	203.51	57.25

The median duration for the rat survived after suffering shock was 32.4 minutes. The maximum time for which any rat survived in this group was a little over one hour (62.3 minutes), and the quickest any animal died was within 10 minutes.

***Table 8*: This is from Group 2, n =7. The time taken for death to occur after 50% drop in MAP is evaluated here.**

Effect of noradrenaline Descriptive statistics		**Time to MAP drop (in minutes)**	**Time interval from MAP drop to Death (in minutes)**
Total no. (n)		7	7
Mean		96.348	217.60
Median		90.426	170.25
Std. Deviation		28.6356	142.44
Minimum		67.8	104.47
Maximum		151.2	496.04
Percentiles	*25th*	75.666	114.31
	50th	90.426	170.25
	75th	116.112	321.16

7.3. Effect of noradrenaline:

In group 2, after the addition of the organism, when the MAP dropped 50% below the pre-*E. coli* value, infusion of Noradrenaline was started. TABLE 8 shows the results. The median survival time of the rats that were treated with Noradrenaline following the morbid decline in the MAP improved to about ~170 minutes, i.e. more 2 hours than that of the group-1 rats. The maximum survival time was 496 minutes (~8.27 hours) and the minimum survival time was 104 minutes.

The observed time difference among the two groups (1 & 2) was significant ($p < 0.05$) (see table 9 for exact p value), compared using nor-parametric test Mann-Whitney U and Wilcoxon signed-rank tests. Figure 42, shows the difference in the survival time graphically.

***Table 9*: Statistical analysis to find out if the difference between the survival time in Group 1 & 2.**

Difference between survival time.	**Time to death (in Minutes)**
Mann-Whitney U	.000
Wilcoxon W	28.000
Z	-3.130
Asymp. Sig. (2-tailed)	.002
Exact Sig. [2*(1-tailed Sig.)]	.001[a]

a. Not corrected for ties.

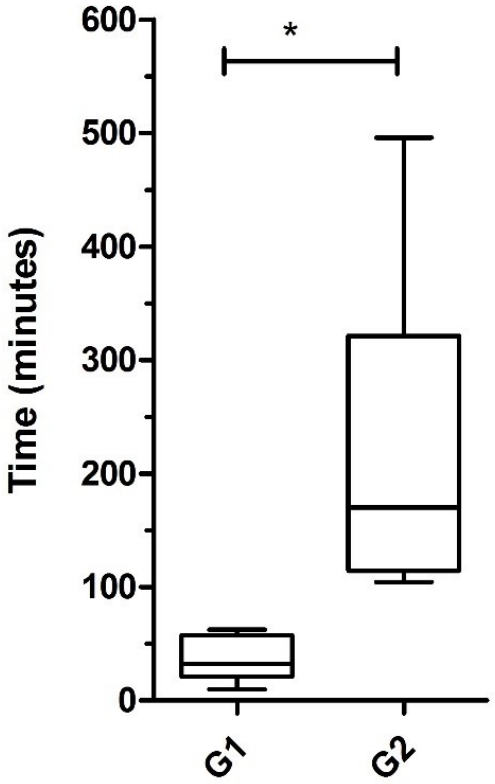


***Figure 39*: Comparison of the survival time after 50% drop in MAP among the two groups (1 & 2). * significant difference (p <0.05).**

7.4. Other physiological parameters:

Cardio-vascular parameters like Systolic, Diastolic and Mean arterial pressures, the heart rate, and pulse pressure were also studied.

a. *Systolic, Diastolic, and MAP*: In the first group, there was a significant drop in the Systolic, Diastolic, and mean arterial pressures. The details are represented graphically in the FIGURE 43.

E. coli decreased the heart rate and widened the pulses pressure as the septic shock progressed to causing death.

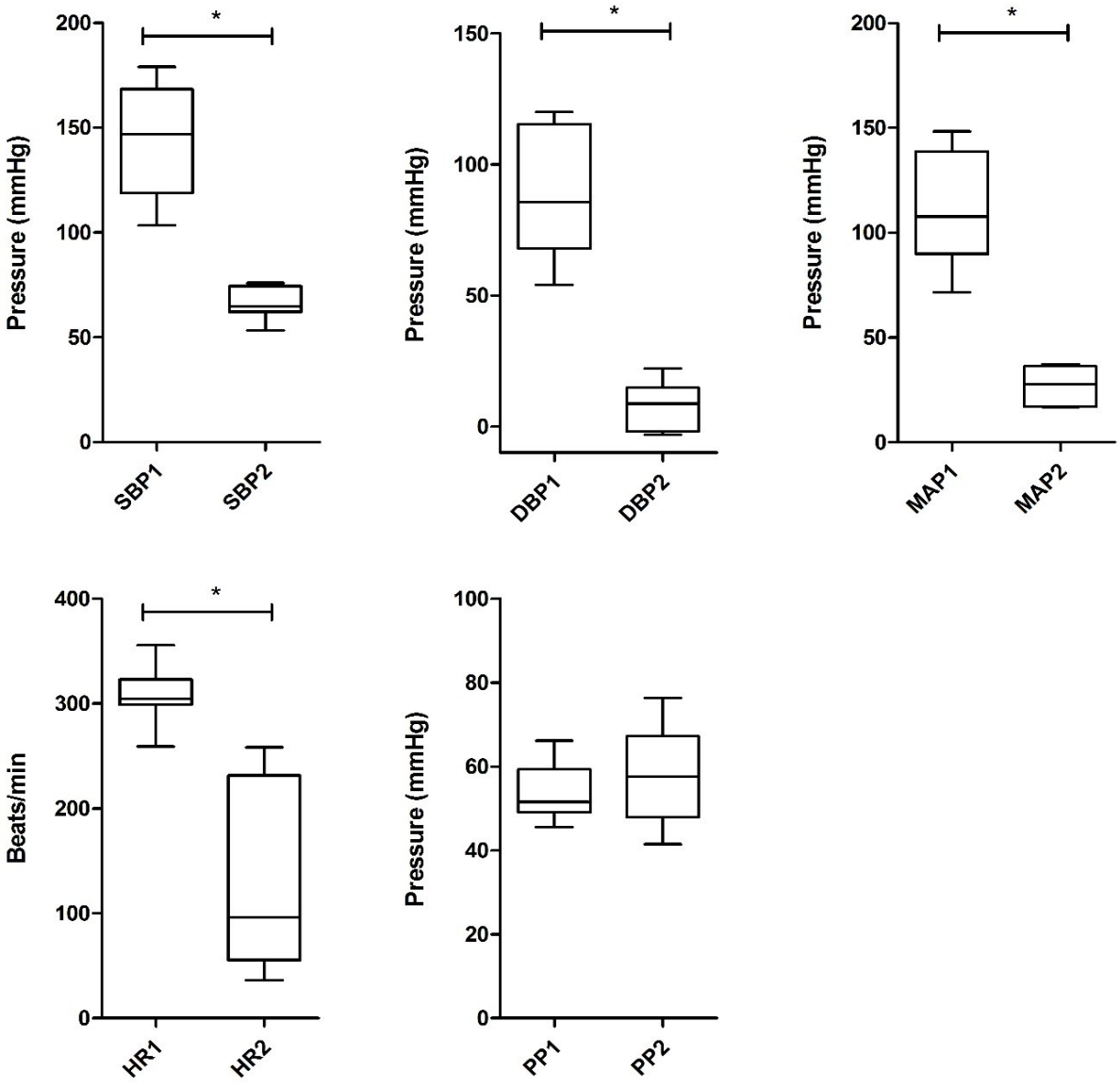


***Figure 40*: This is from Group 1, n =7. Systolic (SBP), Diastolic (DBP), Mean arterial Pressure (MAP), Hear rate (HR), Pulse Pressure (PP) are compared and plotted. 1 – before addition of *E. coli*; 2 – after adding *E. coli*.**

b. *SBP, DBP, MAP, HR and PP in Group 2*: Comparison of the various aspects of pressure in all the three stages of Group 2 –

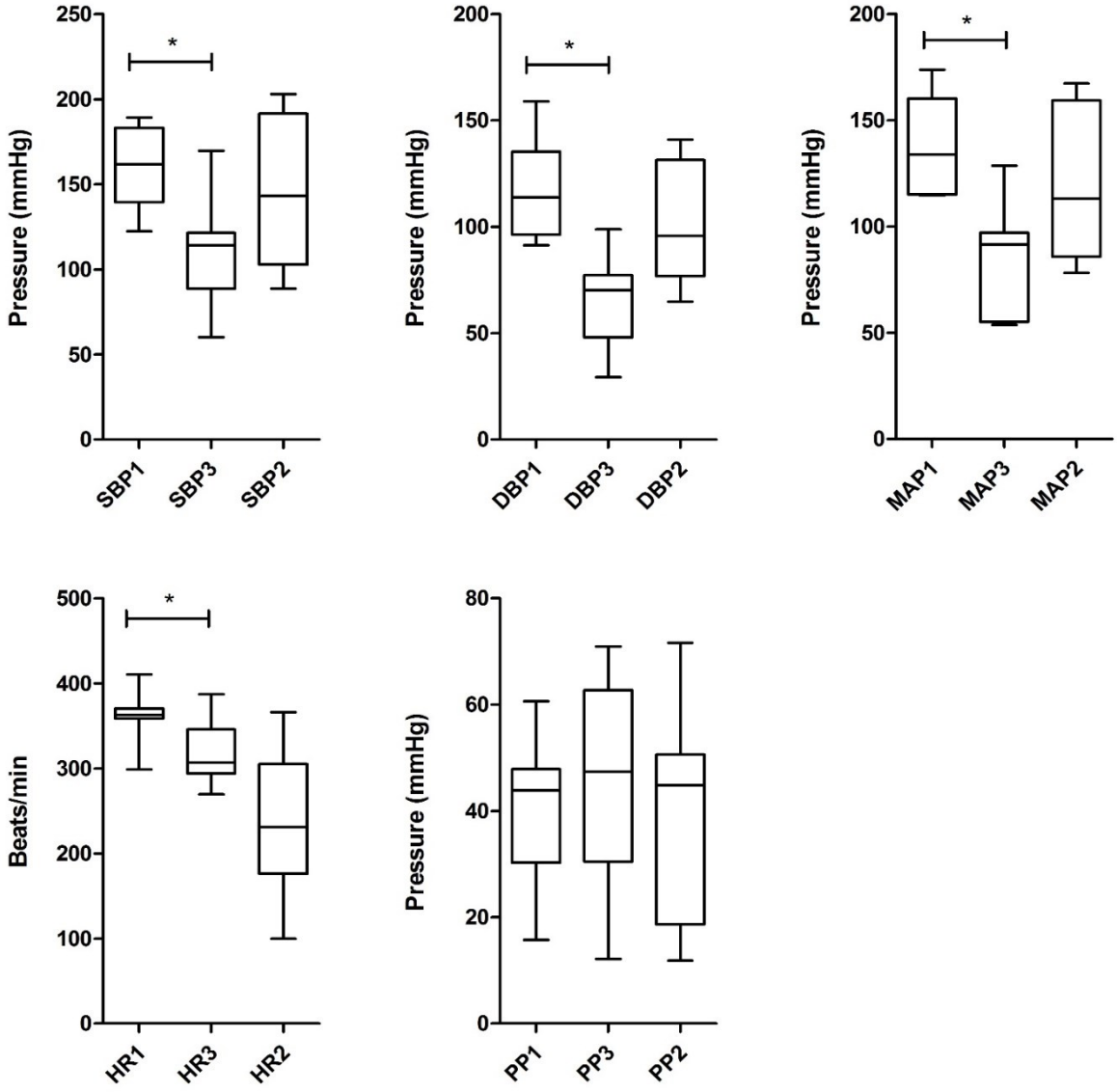


***Figure 41*: This is from Group 2 (*E. coli* + Noradrenaline), n =7. Systolic (SBP), Diastolic (DBP), Mean arterial Pressure (MAP), Hear rate (HR), Pulse Pressure (PP) are compared and plotted. 1 – before addition of *E. coli*; 3 – before the intervention when the rat is in septic shock due to *E. coli*.; 2 – after intervention with Noradrenaline.**

Before *E. coli*, After *E. coli*, i.e. development of shock before Intervention and after intervention (figure 44).

c. *The control group*, where only normal saline was injected was evaluated for all the parameters. These rats were euthanized

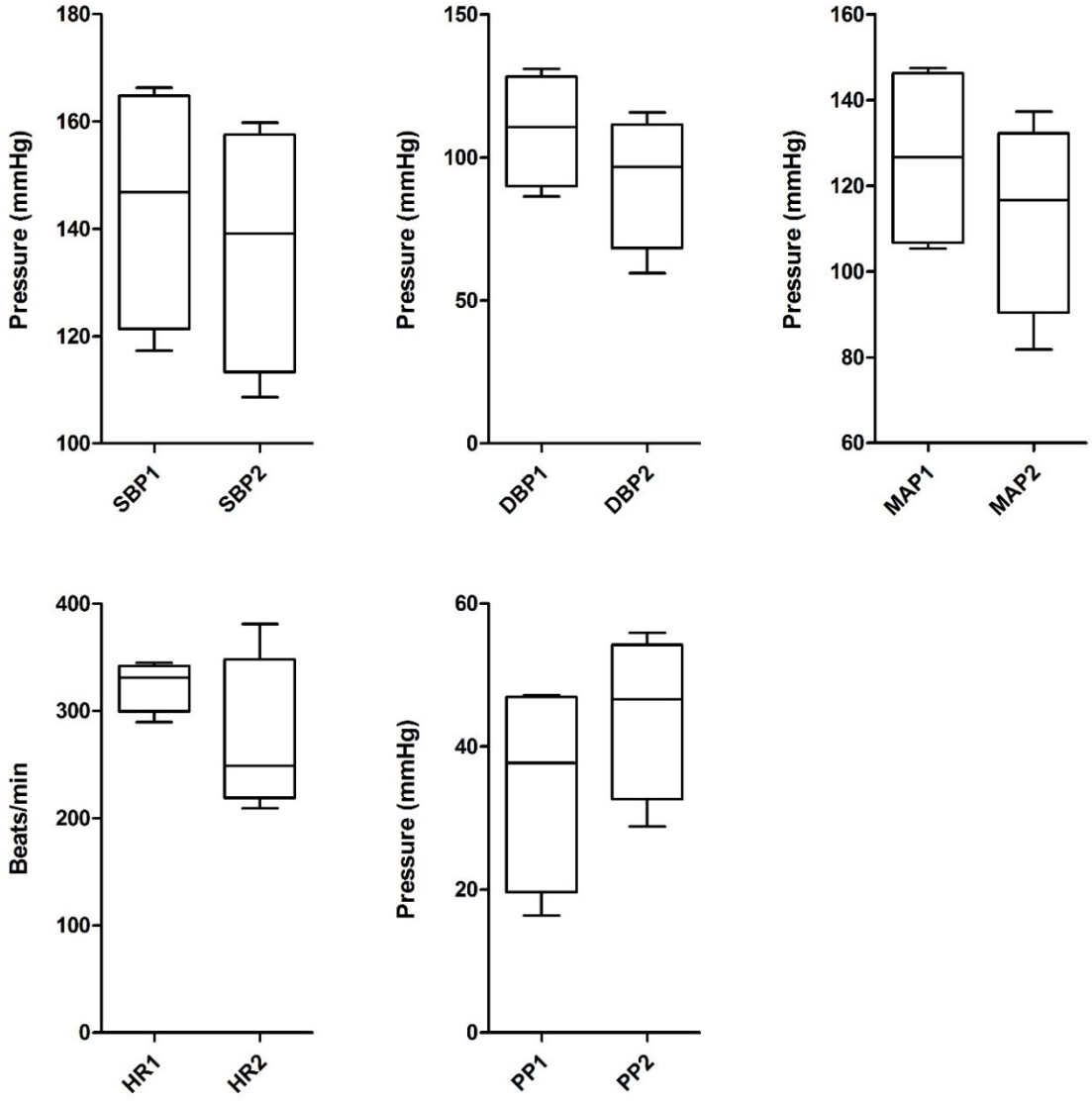


***Figure 42*: This is from Group 3, n =4. Systolic (SBP), Diastolic (DBP), Mean arterial Pressure (MAP), Hear rate (HR), Pulse Pressure (PP) are compared and plotted. 1 – At start of Experiment; 2 – At end of experiment.**

after the end of 8 hours of experiment (figure 45).

CHAPTER 8 DISCUSSION

8.1. Objective 1: Creating acute and stable model of septic shock in adult Wistar rats.

In this study, we started out trying to make a model of septic shock in Wistar rats using strains of live *E. coli* bacilli. To do so, we have initially diverted our attention to perfecting the art of carotid cannulation. In order to keep the rat under anesthesia for the whole duration of the experiment, we tried, successfully, to cannulate the lateral tail vein. This helped us achieve continuous IV access that enabled us to give the cocktails of anesthetics (Ketamine + Midazolam) and additional supply of IV-fluids. All these enable a pain-free environment for animal experiments and reduces the suffering to the animal.

E. coli was also obtained from the department of microbiology freely as it was regularly prepared for antibiotic testing. This reduces the cost by a considerable amount, unlike the use of lipopolysaccharides (endotoxemia model) that costs several thousands of rupees. The quantity of *E. coli* dose needed to create septic shock was done in prior standardizations experiments. Aiding in a semi-quantitative estimation of the amount of *E. coli* to be added was the McFarland standards that were easy to prepare in our laboratory.

E. coli dose was capped at 650 μL/100g of 5 McFarland unit turbidity, which was administered IV through the tail vein. This, as we saw, produced a 50% drop in the mean arterial pressure from the initial value in about 75% of the

animals (n=14) by 2.5 hours (max – 4.2 hrs & min – 0.6hrs). Once the animal reached a MAP 50% lesser than their pre-*E. coli* state, 75% died of them died within an hour (max – 62.3 minutes & min – 9.9 minutes). There is also clear evidence of biological variability seen in the way different animals respond to the *E. coli* and what different *E. coli* inoculum might behave.

8.2. Objective 2: Effect of Noradrenaline:

Noradrenaline is a potent non-specific adrenergic agonist drug known to cause vasoconstriction and increase the heart rate. Therefore, it must, in this case must cause vaso-constriction and pick up the fall in blood pressure.

We first calculated the dose of noradrenaline for the rat and loaded it in the infusion pump to last for the 7-8 hours of infusion. It was evident that noradrenaline prolonged life of the rat as evidenced by the significant rise in the time to death from the time when the MAP dropped to 50% of its initial value (Mann-Whitney U test was used, Z value was -3.130, Asymp. Sig. (2-tailed) .002).

Noradrenaline had a positive effect on the systolic diastolic and mean arterial pressures though the differences were not found to be significant. As in the case of pulse pressure, there was a widening that was observed with the progress of sepsis which was not reversed, and in some case it widened. The effect of noradrenaline on the heart rate was found to be ambivalent, and a comparison of the values from groups 1 & 2 (i.e., test and intervention), before and after the administration of the drug was not significant.

The median survival time for Group1 was 32.4 minutes, and that of Group2 was 170.3 minutes. So, there was a $170.3/32.4 = 5.3$ times increase in the median survival time. The time of survival was found to be significantly different, $p = 0.001$ using the Mann-Whitney U test.

CHAPTER 9 CONCLUSION

The setting up of an animal model to induce septic shock by injection of live *E. coli* containing inoculum is possible. We have been successful in demonstrating a significant drop in all the components of blood pressure – Systolic, Diastolic, and MAP. There was a significant decline in the heart rate and widening of the pulse pressure.

Noradrenaline proved beneficial in septic shock as it increased the survival time even after the animal developed a MAP 50% less than its initial value. It improved the systolic diastolic and mean arterial pressures but no significant improvement in the heart rate or pulse pressure.

CHAPTER 10 LIMITATIONS & FUTURE PROSPECTS

10.1. LIMITATIONS

The study is limited to only blood pressure measurements.

It needs a larger sample for concluding with a higher degree of certainty.

10.2. FUTURE PROSPECTS

1. The above model provides scope for further studies on the various physiological studies on sepsis.
2. Moreover, experiments can be planned with sublethal doses of the inoculum that can be used for studying antibiotic sensitivity and resistance testing.
3. Further exploration concerning other parameters like blood pressure and heart rate variability is possible.
4. The interactions in endocrine cardiovascular hematological axis can be studied further.
5. Septic models from other commonly related organisms that are most commonly encountered in clinical practice can be taken up in the future.

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CHAPTER 12 APPENDIX

IRB Approval

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Anna Benjamin Pulimood, M.B.B.S., MD., Ph.D.,
Chairperson, Research Committee & Principal

Dr. Biju George, M.B.B.S., MD., DM.,
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

January 30, 2018

Dr. Sajo Thomas,
PG Registrar,
Department of Physiology,
Christian Medical College,
Vellore – 632 002.

Sub: **Fluid Research Grant: New Proposal:**
A rat model for septic shock using strains of bacteria to study the effects of alpha adrenergic receptor stimulant and blocker.
Dr. Sajo Thomas (emp. No. 21470), Physiology, Dr. Sathya Subramani (Emp. No. 14123), Physiology, Dr. Aravindhan V (Emp. No. 21549), PG Registrar, Physiology.

Ref: IRB Min. No. 11033 [OTHER] dated 04.12.2017

Dear Dr. Sajo Thomas,

The Institutional Review Board **(Blue**, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project titled "A rat model for septic shock using strains of bacteria to study the effects of alpha adrenergic receptor stimulant and blocker" on December 04th 2017.

The Committee reviewed the following documents:

1. IRB application format
2. Cvs of Drs. Sajo Thomas, Sathya Subramani, Aravindhan V
3. No. of documents 1- 2.

The following Institutional Review Board (Blue, Research & Ethics Committee) members were present at the meeting held on December 04th 2017 in the CK Job Hall, Paul Brand Building, Christian Medical College, Vellore 632 004.

2 of 4

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Anna Benjamin Pulimood, M.B.B.S., MD., Ph.D.,
Chairperson, Research Committee & Principal

Dr. Biju George, M.B.B.S., MD., DM.,
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Name	Qualification	Designation	Affiliation
Dr. Biju George	MBBS, MD, DM	Professor, Haematology, Research), Additional Vice Principal , Deputy Chairperson (Research Committee), Member Secretary (Ethics Committee), IRB, CMC, Vellore	Internal, Clinician
Dr. Anuradha Rose	MBBS, MD, MHSC (Bioethics)	Associate Professor, Community Health, CMC, Vellore	Internal, Clinician
Dr. Thomas V Paul	MBBS, MD, DNB, PhD	Professor, Endocrinology, CMC, Vellore	Internal, Clinician
Dr. RekhaPai	BSc, MSc, PhD	Associate Professor, Pathology, CMC, Vellore	Internal, Basic Medical Scientist
Rev. Joseph Devaraj	BSc, BD	Chaplaincy Department, CMC, Vellore	Internal, Social Scientist
Mr. Samuel Abraham	MA, PGDBA, PGDPM, M. Phil, BL.	Sr. Legal Officer, CMC, Vellore	Internal, Legal Expert
Mr. C. Sampath	BSc, BL	Advocate, Vellore	External, Legal Expert
Ms. Grace Rebekha	M.Sc., (Biostatistics)	Lecturer, Biostatistics, CMC, Vellore	Internal, Statistician
Dr Sneha Varkki	MBBS, DCH, DNB	Professor, Paediatrics, CMC, Vellore	Internal, Clinician
Dr. Sowmya Sathyendra	MBBS, MD (Gen. Medicine)	Professor, Medicine III, CMC, Vellore	Internal, Clinician
Dr. Asha Solomon	MSc Nursing	Associate Professor, Medical Surgical Nursing, CMC, Vellore	Internal, Nurse

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Anna Benjamin Pulimood, M.B.B.S., MD., Ph.D.,
Chairperson, Research Committee & Principal

Dr. Biju George, M.B.B.S., MD., DM.,
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Dr. Sathish Kumar	MBBS, MD, DCH	Professor, Child Health, CMC, Vellore	Internal, Clinician
Mrs. Emily Daniel	MSc Nursing	Professor, Medical Surgical Nursing, CMC, Vellore	Internal, Nurse
Dr. Mathew Joseph	MBBS, MCH	Professor, Neurosurgery, CMC, Vellore	Internal, Clinician
Dr. Shyam Kumar NK	MBBS, DMRD, DNB, FRCR, FRANZCR	Professor, Radiology, CMC, Vellore	Internal, Clinician
Mrs. Pattabiraman	BSc, DSSA	Social Worker, Vellore	External, Lay Person
Mrs. Sheela Durai	MSc Nursing	Professor, Medical Surgical Nursing, CMC, Vellore	Internal, Nurse
Dr. John Antony Jude Prakash	MBBS, MD	Professor, Clinical Microbiology, CMC, Vellore.	Internal, Clinician.

We approve the project to be conducted as presented.

Kindly provide the total number of patients enrolled in your study and the total number of Withdrawals for the study entitled: "A rat model for septic shock using strains of bacteria to study the effects of alpha adrenergic receptor stimulant and blocker" on a monthly basis. Please send copies of this to the Research Office (research@cmcvellore.ac.in).

Fluid Grant Allocation:
A sum of 1,00,000/- INR (Rupees One Lakh Only) will be granted for 2 years. 50,000/- INR (Rupees Fifty Thousand only) will be granted for 12 months as an 1st Installment. The rest of the 50,000/- INR (Rupees Fifty thousand only) each will be released at the end of the first year as 2 nd Installment.

Yours sincerely,

Dr. Biju George
Secretary (Ethics Committee)
Institutional Review Board

Dr. BIJU GEORGE
MBBS., MD., DM.
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

IRB Min. No. 11033 [OTHER] dated 04.12.2017

Animal Ethics approval:

INSTITUTIONAL ANIMAL ETHICS COMMITTEE
CHRISTIAN MEDICAL COLLEGE, VELLORE

Dr. Anna B. Pulimood
Principal and Chairperson
email: princi@cmcvellore.ac.in

Dr. Joe Varghese
Secretary, IAEC, CMC, Vellore
email: joevarghese@cmcvellore.ac.in

Date: 26th March 2018

Dr. Sajo Thomas,
PG Registrar,
Department of Physiology,
CMC, Vellore.

Dear Dr. Sajo Thomas,

Your research proposal titled "A rat model for septic shock using strains of bacteria to study the effects of alpha adrenergic receptor stimulant and blocker" has been reviewed by the Institutional Animal Ethics Committee (IAEC) at the meeting held on 26.02.2018.

After discussion, **14 (fourteen) Wistar rats** have been approved for the study for a period of **12 months.**

The IAEC approval number for the study is **8/2018.**

As per the requirements of the CPCSEA, you will have to maintain all records as per Form D and ensure humane treatment of animals. For sanction of animals for the 2nd year of the project, a request for the same along with a **progress report** must be submitted to the IAEC.

It is the responsibility of the Principal Investigator to ensure that all experiments on animals are conducted as per the proposal submitted and approved by the IAEC. No deviation from the approved proposal is allowed unless the approval of the IAEC for such deviation(s) is requested and granted.

With best wishes,

Yours sincerely,

Dr. Anna Pulimood,
Principal and Chairperson (IAEC)
CMC Vellore

04/04/2018
Dr. A. Yasotha
CPCSEA – Main Nominee

McFarland Standard

McFARLAND STANDARD

-For in vitro use only- Catalogue No. TM50-TM60

McFarland Standards are used to standardize the approximate number of bacteria in a liquid suspension by comparing the turbidity of the test suspension with that of the McFarland Standard.

A McFarland Standard is a chemical solution of barium chloride and sulfuric acid; the reaction between these two chemicals results in the production of a fine precipitate, barium sulfate. When shaken well, the turbidity of a McFarland Standard is visually comparable to a bacterial suspension of known concentration as indicated below.

Cat No.	McFarland Standard	1% $BaCl_2$ (mL)	1% H_2SO_4 (mL)	Approximate Bacterial Suspension / mL
TM50	0.5	0.05	9.95	1.5×10^8
TM51	1.0	0.10	9.90	3.0×10^8
TM52	2.0	0.20	9.80	6.0×10^8
TM53	3.0	0.3	9.7	9.0×10^8
TM54	4.0	0.4	9.6	1.2×10^9
TM55	5.0	0.5	9.5	1.5×10^9
TM56	6.0	0.6	9.4	1.8×10^9
TM57	7.0	0.7	9.3	2.1×10^9
TM58	8.0	0.8	9.2	2.4×10^9
TM59	9.0	0.9	9.1	2.7×10^9
TM60	10.0	1.0	9.0	3.0×10^9

Prior to using, the McFarland Standard should be shaken up well and aliquoted into test tubes identical to those used to prepare the inoculum suspension. Once aliquoted the tubes should be tightly sealed to prevent evaporation from occurring. Before each use, shake well to ensure that the barium sulfate is distributed evenly throughout the solution.

The standard most commonly used in the clinical microbiology laboratory is the 0.5 McFarland Standard, which is prescribed for antimicrobial susceptibility testing and culture media performance testing.

Procedures

1. Mix McFarland Standard on a vortex mixture prior to examination. Ensure that the McFarland Standard is aliquoted into a tube that is the same size and diameter as the tube used to prepare the test suspension.
2. Prepare a test suspension by obtaining a fresh, pure culture of the test organism and inoculating a suitable broth.
3. In the presence of good lighting, visually compare the turbidity of test suspension with that of the McFarland standard by comparing the clarity of the lines on the a Wickerham card.
4. If the test suspension is too light, inoculate with additional organisms or incubate tube until turbidity matches that of the standard. If dilution is necessary, use a sterile pipette and add sufficient broth or saline to obtain a turbidity that matches that of the standard.

Precautions

- *McFarland Standards are sensitive to air and light therefore ensure that the tubes are closed tightly at all times and kept in the dark*
- *The level of McFarland standards should be checked occasionally to ensure that evaporation has not occurred. Discard if any volume is lost*
- *The McFarland Standards should be vigorously agitated on a mechanical vortex before each use and inspected for a uniform turbid appearance. If large particles appear or if clumping is apparent discard the standard*
- *Actual numbers of viable bacteria present in an adjusted suspension depends on the size, viability, and clumping of the particular bacterium used*

Statistics

Test Statistics[a]

	SBP_3 - SBP_1	DBP_3 - DBP_1	MAP_3 - MAP_1	HR _3 - HR _1
Z	-2.366[b]	-2.366[b]	-2.366[b]	-2.366[b]
Asymp. Sig. (2-tailed)	.018	.018	.018	.018

	PP_3 - PP_1
Z	-1.014[c]
Asymp. Sig. (2-tailed)	.310

a. Wilcoxon Signed Ranks Test
b. Based on positive ranks.
c. Based on negative ranks.

Wilcoxon Signed Ranks Test

Within Group 2 (NorAd)

Test Statistics[a]

	SBP_2 - SBP_1	DBP_2 - DBP_1	MAP_2 - MAP_1	HR_2 - HR _1
Z	-1.352[b]	-1.521[b]	-1.521[b]	-2.366[b]
Asymp. Sig. (2-tailed)	.176	.128	.128	.018

Test Statistics[a]

	PP_2 - PP_1	SBP_3 - SBP_2	DBP_3 - DBP_2	MAP_3 - MAP_2
Z	-.338[b]	-1.859[b]	-1.859[b]	-1.859[b]
Asymp. Sig. (2-tailed)	.735	.063	.063	.063

Test Statistics[a]

	HR _3 - HR_2	PP_3 - PP_2
Z	-1.859[c]	-.845[c]
Asymp. Sig. (2-tailed)	.063	.398

a. Wilcoxon Signed Ranks Test
b. Based on positive ranks.
c. Based on negative ranks.

Statistics

GROUP			SBP_1	DBP_1	MAP_1	HR _1	PP_1
1	N	Valid	7	7	7	7	7
		Missing	0	0	0	0	0
	Mean		144.9252	90.4488	114.3179	309.3699	54.4759
	Median		146.9765	85.7017	107.6215	304.4875	51.5466
	Std. Deviation		27.23703	25.12414	28.53858	29.48573	7.19951
	Minimum		103.26	54.13	71.58	259.10	45.53
	Maximum		178.97	120.04	148.33	355.58	66.19
	Percentiles	25	118.7104	67.9738	89.9012	299.0049	49.1332
		50	146.9765	85.7017	107.6215	304.4875	51.5466
		75	168.3302	115.4428	138.7283	323.0913	59.2616
2	N	Valid	7	7	7	7	7
		Missing	0	0	0	0	0
	Mean		160.8723	119.5632	138.5431	360.9917	41.3098
	Median		161.5837	113.8036	133.9860	362.9218	43.8707
	Std. Deviation		24.57373	24.35487	24.08347	32.77822	14.39411
	Minimum		122.47	91.32	114.53	298.59	15.76
	Maximum		189.24	158.99	173.74	410.44	60.65
	Percentiles	25	139.3608	96.3498	115.1214	358.6778	30.2574
		50	161.5837	113.8036	133.9860	362.9218	43.8707
		75	183.1415	135.3062	160.1422	370.4507	47.8370
3	N	Valid	4	4	4	4	4
		Missing	0	0	0	0	0
	Mean		144.3412	109.5743	126.5162	324.0513	34.7667

	Median		146.8397	110.5736	126.6325	330.9696	37.7360
	Std. Deviation		22.96800	19.84138	21.52145	23.96219	14.73672
	Minimum		117.32	86.32	105.34	289.57	16.39
	Maximum		166.37	130.83	147.46	344.70	47.20
	Percentiles	25	121.3695	89.9686	106.7006	299.3213	19.6239
		50	146.8397	110.5736	126.6325	330.9696	37.7360
		75	164.8143	128.1805	146.2155	341.8630	46.9401

Statistics

Descriptive statistics within individual groups

Group 1 → Only E. coli

Group 2 → E. coli **&** Intervention

Group 3 → Negative control

{SBP2, DBP2, MAP2, HR2, PP2} → **After E. coli OR Intervention**

GROUP			SBP_2	DBP_2	MAP_2	HR_2	PP_2
1	N	Valid	7	7	7	7	7
		Missing	0	0	0	0	0
	Mean		65.8442	7.9044	26.8582	125.9011	57.9414
	Median		64.6282	8.7527	27.6628	96.4027	57.6018
	Std. Deviation		7.69692	8.92852	9.23053	85.39577	12.34516
	Minimum		53.22	-3.13	16.78	36.24	41.49
	Maximum		75.95	22.12	37.19	258.30	76.31
	Percentiles	25	62.1443	-1.9438	17.0639	55.4676	47.9183
		50	64.6282	8.7527	27.6628	96.4027	57.6018
		75	74.3704	14.8262	36.3163	231.3883	67.1950
2	N	Valid	7	7	7	7	7
		Missing	0	0	0	0	0
	Mean		140.3051	99.1934	116.8346	241.8349	41.1116
	Median		143.1448	95.6954	113.1184	231.1302	44.8134
	Std. Deviation		44.17833	27.95398	34.97817	88.58755	20.15860
	Minimum		88.78	64.84	78.15	99.57	11.87
	Maximum		202.93	140.95	167.32	366.25	71.63
	Percentiles	25	102.8535	76.9073	85.7244	176.1927	18.6452
		50	143.1448	95.6954	113.1184	231.1302	44.8134
		75	191.5707	131.2948	159.3041	305.0435	50.6275
3	N	Valid	4	4	4	4	4
		Missing	0	0	0	0	0
	Mean		136.6708	92.1707	113.0784	271.9571	44.4977
	Median		139.1115	96.7241	116.6051	248.8535	46.6099
	Std. Deviation		23.12594	23.58604	23.02585	75.00807	11.51278
	Minimum		108.65	59.52	81.80	209.20	28.84
	Maximum		159.81	115.71	137.31	380.92	55.93
	Percentiles	25	113.3497	68.3484	90.4025	219.0041	32.6514
		50	139.1115	96.7241	116.6051	248.8535	46.6099
		75	157.5510	111.4398	132.2276	348.0137	54.2318

Statistics

Descriptive statistics within individual groups

Group 1 → Only E. coli

Group 2 → E. coli **&** Intervention

Group 3 → Negative control

{SBP3, DBP3, MAP3, HR3, PP3} → **Before Intervention**

2	N	Valid	7	7	7	7	7
		Missing	0	0	0	0	0
	Mean		111.3055	64.5705	84.7981	318.3661	46.7344
	Median		114.0840	70.1119	91.4744	306.6429	47.3663
	Std. Deviation		33.38582	22.59283	26.54198	38.68570	20.28122
	Minimum		60.18	29.35	53.78	269.53	12.21
	Maximum		169.67	98.72	128.76	387.32	70.95
	Percentiles	25	88.7638	47.9710	55.1168	294.0189	30.4741
		50	114.0840	70.1119	91.4744	306.6429	47.3663
		75	121.4120	77.2187	97.1414	345.8387	62.7594

GROUP			Time to MAP drop (in Hrs)	Time to death (in Minutes)
1	N	Valid	7	7
		Missing	0	0
	Mean		2.2739	38.2422
	Median		2.1990	32.3866
	Std. Deviation		1.24618	20.56462
	Minimum		.60	9.90
	Maximum		4.22	62.30
	Percentiles	25	1.3791	21.0552
		50	2.1990	32.3866
		75	3.3919	57.2468
2	N	Valid	7	.0027
		Missing	0	0
	Mean		1.6058	217.5971
	Median		1.5071	170.2500
	Std. Deviation		.47726	142.43716
	Minimum		1.13	104.47
	Maximum		2.52	496.04
	Percentiles	25	1.2611	114.3100
		50	1.5071	170.2500
		75	1.9352	321.1600

Matlab code used here:

Animal Model for Septic Shock

The data generated by the experiments done on ***Wistar Rats*** by measuring the carotid artery pressures using an *Ipex*® pressure transducer connected to an amplifier which is calibrated (the procedure is mentioned elsewhere), along with the respiration and ECG are all recorded using *CMCDAQ*® or *AD Instruments® Lab Chart pro software.* This data is later filtered and analysed using Matlab® codes. This is the codes for running data collected from LabChart Pro®.

Import file through UI

```
% [~,F_name,~] =
fileparts(uigetfile('*.mat'));
% load(F_name);
[F_name,F_Path] =
uigetfile('*.mat',...
    'File FOR ECOLI - 1v (1st
version)');
load(F_name);
F = fullfile(F_Path,F_name);
[F_Path,F_name,F_ext] = fileparts(F);
N_dir = sprintf('%s',F_name) ;
mkdir(N_dir)
N_dir = cd(N_dir);
```

Special Considerations

For 190213_WR_SS including the initial segment

```
%{
S1a2 =
[data(datastart(1,1):dataend(1,1)),..
.         % segments 1 and 2
data(datastart(1,2):dataend(1,2))];
S1a2n = S1a2-250;
ndata =
[S1a2n,data(datastart(1,3):dataend(1,
3));];
data = ndata;
%}
```

Make the selection of the graph

First find the location of the comment(s) in the data. If there are more than one segments in the recording the values (location) of the comments will change when the whole data is taken together as the location of each segment starts from zero.

```
if size(datastart,2) > 1
    ncom = zeros(size(com,1),2);
      for c = 1:size(com,1)
         if com(c,2) == 1
             ncom(c,1) = com(c,3);
          elseif com(c,2) > 1
           ncom(c,1) =
com(c,3)+dataend(com(c,2)+com(c,1));
         end
       end
  ncom(:,2) = com(:,5);

  X1 =
contains(cellstr(comtext),'Selec 1');
  loc1 = find(X1);
  X11 = ismember(ncom,loc1);
  loc11 = find(X11)-size(com,1);
  Selec1 = ncom(loc11,1);

  X2 =
contains(cellstr(comtext),'Selec 2');
  loc2 = find(X2);
  X22 = ismember(ncom,loc2);
  loc22 = find(X22)-size(com,1);

  Selec2 = ncom(loc22,1);

 end

if size(datastart,2) == 1
    X1 =
contains(cellstr(comtext),'Selec 1');
     loc1 = find(X1);
    X2 =
contains(cellstr(comtext),'Selec 2');
     loc2 = find(X2);

    Selec1 = com(loc1,3);
    Selec2 = com(loc2,3);
end
```

Pick the selected data 'Selec1' & 'Selec2'

Using the locations found in the previous section pick and store the data into new variables. This is then plotted to see the difference.

```
Selec1Data =
data(Selec1:Selec1+60000);
Selec2Data =
data(Selec2:Selec2+60000);
```

Finding Peaks and Troughs

1. Find peaks - systolic and diastolic of '**Selec1Data**'

```
% Systolic
figure('Visible','on')
findpeaks(Selec1Data,'minpeakprominen
ce',25, 'minpeakdistance', 150)     %
"will need change - be careful"
```

```
[sbp1,sbp1loc] =
findpeaks(Selec1Data,'minpeakprominen
ce',25,...
    'minpeakdistance', 150);
pause
% Diastolic
figure('Visible','on')
findpeaks(-
Selec1Data,'minpeakprominence',10,
'minpeakdistance', 100)     % "will
need change - be careful"
[dbp1,dbp1loc] = findpeaks(-
Selec1Data,'minpeakprominence',10,...
    'minpeakdistance', 150);
dbp1 = -dbp1;
pause
close all
```

2. Find peaks - systolic and diastolic of '**Selec2Data**'

```
% Systolic
figure('Visible','on')
findpeaks(Selec2Data,'minpeakprominen
ce',25, 'minpeakdistance', 150)  %
"will need change - be careful"
[sbp2,sbp2loc] =
findpeaks(Selec2Data,'minpeakprominen
ce',25,...
    'minpeakdistance', 150);
pause
% Diastolic
figure('Visible','on')
findpeaks(-
Selec2Data,'minpeakprominence',20,...
    'minpeakdistance', 150)   % "will
need change - be careful"
[dbp2,dbp2loc] = findpeaks(-
Selec2Data,'minpeakprominence',20,...
    'minpeakdistance', 150);
dbp2 = -dbp2;
pause
close all
```

3. Pair the data of '**Selec1Data**' - [sbp1] [sbp1loc] [dbp1] [dbp1loc]

```
n1 = min(size(dbp1,2),size(sbp1,2));
bppairs1 = zeros(4,n1); %
bppairs1(dbp1loc,dbp1,sbp1loc,sbp1)
        for m1 = 1:n1
            x1 =
find(dbp1loc<sbp1loc(m1));
            if isempty(x1) || ...
               sbp1loc(m1)-
dbp1loc(max(x1)) > 100
                    bppairs1(1,m1) =
0;
                    bppairs1(2,m1) =
0;
                    bppairs1(3,m1) =
0;
                    bppairs1(4,m1) =
0;
            else
                    bppairs1(1,m1) =
dbp1loc(max(x1));
                    bppairs1(2,m1) =
dbp1(max(x1));
                    bppairs1(3,m1) =
sbp1loc(m1);
                    bppairs1(4,m1) =
sbp1(m1);
            end
        end
bppairs1 = bppairs1'; % now becomes a
column vectors
bppairs1(dbp1loc,dbp1,sbp1loc,sbp1)
bppairs1(~any(bppairs1,2),:) = [];
```

4. Pair the data of 'Selec2Data' - [dbp2loc] [dbp2] [sbp2loc] [sbp2];

```
n2 = min(size(dbp2,2),size(sbp2,2));
bppairs2 = zeros(4,n2); %
bppairs1(dbp1loc,dbp1,sbp1loc,sbp1)
        for m2 = 1:n2
            x2 =
find(dbp2loc<sbp2loc(m2));
            if isempty(x2) || ...
               sbp2loc(m2)-
dbp2loc(max(x2)) > 190  % "will need
change"
                    bppairs2(1,m2) =
0;
                    bppairs2(2,m2) =
0;
                    bppairs2(3,m2) =
0;
                    bppairs2(4,m2) =
0;
            else
                    bppairs2(1,m2) =
dbp2loc(max(x2));
                    bppairs2(2,m2) =
dbp2(max(x2));
                    bppairs2(3,m2) =
sbp2loc(m2);
                    bppairs2(4,m2) =
sbp2(m2);
            end
        end
bppairs2 = bppairs2'; % now becomes a
column vectors
bppairs2(dbp2loc,dbp2,sbp2loc,sbp2)
bppairs2(~any(bppairs2,2),:) = [];
```

Analysis

1. Mean Systolic and Diastolic and mean arterial pressure (MAP):

```
                                % for
'Selec1Data'
q1 = size(bppairs1,1)-1;
MAPselec1 = zeros(1,q1);
for p1 = 1:q1
    AUC_w1 =
trapz(Selec1Data(bppairs1(p1,1):bppai
rs1(p1+1,1)));
    w1_time = bppairs1(p1+1,1)-
bppairs1(p1,1);
    MAPselec1(p1) = AUC_w1/w1_time;
end
meansbp1 = mean(bppairs1(:,4));
meandbp1 = mean(bppairs1(:,2));
                                % for
'Selec2Data'
q2 = size(bppairs2,1)-1;
MAPselec2 = zeros(1,q2);
for p2 = 1:q2
    AUC_w2 =
trapz(Selec2Data(bppairs2(p2,1):bppai
rs2(p2+1,1)));
    w2_time = bppairs2(p2+1,1)-
bppairs2(p2,1);
    MAPselec2(p2) = AUC_w2/w2_time;
end
meansbp2 = mean(bppairs2(:,4));
meandbp2 = mean(bppairs2(:,2));
```

2. Calculate Heart Rate

First calculate the cycle length and then calculate the difference of the two adjacent location values i.e `sbp1loc` and `sbp2loc`.

```
                                % for
'Selec1Data'
cyclelength_Selec1 =
diff(bppairs1(:,3));
HR_Selec1 =
max(bppairs1(:,3))./cyclelength_Selec
1; % beats per minute
                                % for
'Selec2Data'
cyclelength_Selec2 =
diff(bppairs2(:,3));
HR_Selec2 =
max(bppairs2(:,3))./cyclelength_Selec
2;
```

3. Pulse pressure

```
                                % for
'Selec1Data'
PPSelec1Data = round(bppairs1(:,4)-
bppairs1(:,2),1,'decimals');
                                % for
'Selec2Data'
PPSelec2Data = round(bppairs2(:,4)-
bppairs2(:,2),1,'decimals');
```

Visualization

```
% Confirm points
mygreen = [0 0.6 0.2];
myblue = [0 0.45 0.75];
myred = [0.85 0.33 0.1];
mybrown = [0.64 0.08 0.18];
dotsize = 25;
```

1. Plot "Selec1Data"

```
% Find the max and min values on the
Y axis
maxMap1 = max(bppairs1(:,4));
mM = 900:-100:100;
M1M = find((mM-maxMap1)>0 & (mM-
maxMap1)<100);
yVal1 = mM(M1M)+ 100;
S1 = size(Selec1Data,2);
figure('Visible','on')
plot(Selec1Data,'linewidth',1,...
    'color',mygreen);
hold on
% scatter plot of peaks -
sbp1,sbp1loc; dbp1, dbp1loc over
Selec1Data
scatter(sbp1loc,sbp1,dotsize,'MarkerE
dgeColor',[0 0 0],...
    'MarkerFaceColor',myred);
scatter(dbp1loc(1,:),dbp1(1,:),dotsiz
e,...
    'MarkerEdgeColor',[0 0 0],...
    'MarkerFaceColor',myblue);
xlabel('Time (s)');
ylabel('Pressure (mmHg)');
xlim([0 4000])
ylim([0 yVal1])
line([0 S1],[MAPselec1(1)
MAPselec1(end)],'LineStyle','--',...
    'LineWidth',1,...
    'Color',[0.1 0.1 0.1])
line([0 S1],[meansbp1
meansbp1],'LineStyle','--',...
    'LineWidth',1,...
    'Color',myred)
line([0 S1],[meandbp1
meandbp1],'LineStyle','--',...
    'LineWidth',1,...
    'Color',myblue)
xticks(0:500:4000)
xticklabels(0:0.5:4)
% yticks(0:20:200)
%     [0.190376569037657
0.725783348596814 0.388075313807531
0.187716266935672],...
mystdaxis(gca)
annotation('textbox',...
    [0.142932790645644
0.816774268603874 0.100520835574716
0.0900852896765606],...
```

```
    'String',sprintf('SBP_{mean} =
%.1f mmHg\nMAP_{mean} = %.1f
mmHg\nDBP_{mean} = %.1f mmHg',...

meansbp1,mean(MAPselec1),meandbp1),..
.
        'FitBoxToText','on',...

'HorizontalAlignment','left',...
        'FontName','Georgia',...
        'FontSize',9,...
        'FontAngle','italic',...
        'FontWeight','normal')
annotation('textbox',...

[0.764824308127732,0.836574075315836,
0.127745237992327,0.064814813573051],
...
    'String',sprintf('HR_{avg} =
%.1f/min\nPP_{avg} = %.1f mmHg',...

mean(HR_Selec1),mean(PPSelec1Data)),.
..
        'FitBoxToText','on',...

'HorizontalAlignment','right',...
        'FontName','Georgia',...
        'FontSize',9,...
        'FontAngle','italic',...
        'FontWeight','normal')

Ti1 = title([F_name,' Before
Ecoli'],'Interpreter','none');
mystdpng_ls([F_name,' Before Ecoli'])
```

2. Plot "Selec2Data"

```
% Find the max and min values on the
Y axis
maxMap2 = max(bppairs2(:,4));
mM = 100:100:900;
MM = find(min(abs(mM-maxMap2)));
yVal2 = mM(MM)+ 100;
S2 = size(Selec2Data,2);
figure('Visible','on')
plot(Selec2Data,'linewidth',1,'Color'
,mybrown);
hold on
% scatter plot of peaks -
sbp2,sbp2loc; dbp2, dbp2loc over
Selec1Data
scatter(sbp2loc,sbp2,dotsize,'MarkerE
dgeColor',[0 0 0],...
    'MarkerFaceColor',myred);
scatter(dbp2loc(1,:),dbp2(1,:),dotsiz
e,...
    'MarkerEdgeColor',[0 0 0],...
    'MarkerFaceColor',myblue);
xlabel('Time (s)');
ylabel('Pressure (mmHg)');
xlim([0 4000])
ylim([0 yVal2])
line([0 S2],[MAPselec2(1)
MAPselec2(end)],'LineStyle','--',...
    'LineWidth',1,...
    'Color',[0.1 0.1 0.1])
line([0 S2],[meansbp2
meansbp2],'LineStyle','--',...
    'LineWidth',1,...
    'Color',myred)
line([0 S2],[meandbp2
meandbp2],'LineStyle','--',...
    'LineWidth',1,...
    'Color',myblue)
xticks(0:500:4000)
xticklabels(0:0.5:4)
% yticks(0:20:yVal2)
mystdaxis(gca)
annotation('textbox',...
    [0.142932790645644
0.816774268603874 0.100520835574716
0.0900852896765606],...
    'String',sprintf('SBP_{mean} =
%.1f mmHg\nMAP_{mean} = %.1f mmHg
\nDBP_{mean} = %.1f mmHg',...

meansbp2,mean(MAPselec2),meandbp2),..
.
        'FitBoxToText','on',...

'HorizontalAlignment','left',...
        'FontName','Georgia',...
        'FontSize',9,...
        'FontAngle','italic',...
        'FontWeight','normal');
annotation('textbox',...

[0.764824308127732,0.836574075315836,
0.127745237992327,0.064814813573051],
...
    'String',sprintf('HR_{avg} =
%.1f/min\nPP_{avg} = %.1f mmHg',...

mean(HR_Selec2),mean(PPSelec2Data)),.
..
        'FitBoxToText','on',...

'HorizontalAlignment','right',...
        'FontName','Georgia',...
        'FontSize',9,...
        'FontAngle','italic',...
        'FontWeight','normal');

Ti2 = title([F_name,' After
Ecoli'],'Interpreter','none');
mystdpng_ls([F_name,' After Ecoli'])
```

Saving the required variables in a .mat file

```
% use this to skip the following
section  %{ %}
ReqPar_Selec1 =
{'meansbp1',meansbp1;'meandbp1',meand
bp1;'MAPselec1', mean(MAPselec1);...
    'HR_Selec1',
mean(HR_Selec1);'PPSelec1Data',mean(P
PSelec1Data)};
ReqPar_Selec2 =
{'meansbp2',meansbp2;'meandbp2',meand
bp2;'MAPselec2', mean(MAPselec2);...
    'HR_Selec2',
mean(HR_Selec2);'PPSelec2Data',mean(P
PSelec2Data)};
xlswrite(sprintf('%s.xlsx',F_name),
[ReqPar_Selec1 ReqPar_Selec2]);
save(sprintf('%s_clean.mat',F_name),'
HR_Selec1', 'HR_Selec2',...
    'bppairs1', 'bppairs2',
'MAPselec1', 'MAPselec2',...
    'meandbp1', 'meandbp2',
'meansbp1', 'meansbp2',...
    'PPSelec1Data', 'PPSelec2Data',
'Selec1Data', 'Selec2Data',...
    'F_Path', 'F_name', 'F_ext',
'N_dir');
```

Clear all other variables

```
% clearvars
```

PART 2:

For Plotting the whole experiment. Import file through UI

```
[FF_name,FF_Path] =
uigetfile('*.mat',...
    'File FOR ECOLI - 2v (2nd
version)');
load(FF_name);
FF = fullfile(FF_Path,FF_name);
[FF_Path,FF_name,FF_ext] =
fileparts(FF);
```

Make the selection of the graph

For 190213_WR_SS including the initial segment

```
% use if not using this section
 %{
S1a2 =
[data(datastart(1,1):dataend(1,1)),..
.         % segments 1 and 2

data(datastart(1,2):dataend(1,2))];
S1a2n = S1a2-250;
ndata =
[S1a2n,data(datastart(1,3):dataend(1,
3));];
data = ndata;
%}
```

Seeing the big picture (Seeing the trend of the pressure throughout the whole experiment)

```
fullFdat = data;
%(datastart(1,1):dataend(1,1));
```

Finding Peaks and Troughs - Whole file

1. Find peaks - systolic

```
% Systolic
figure('Visible','on')
hax=axes;
findpeaks(fullFdat,'minpeakprominence
',20, 'minpeakdistance', 150)
[sbpF,sbpFloc] =
findpeaks(fullFdat,'minpeakprominence
',20, 'minpeakdistance', 150);
pause
close all
```

2. Find reverse peaks - diastolic

```
% Diastolic
figure('Visible','on')
findpeaks(-
fullFdat,'minpeakprominence',15,
'minpeakdistance', 100)
[dbpF,dbpFloc] = findpeaks(-
fullFdat,'minpeakprominence',15,
'minpeakdistance', 100);
dbpF = -dbpF;
pause
close all
```

Pair the data of 'Systolic and Diastolic'

- [sbpF] [sbpFloc] [dbpF] [dbpFloc]

```
nF = min(size(dbpF,2),size(sbpF,2));
bppairsF = zeros(4,nF); %
bppairs1(dbp1loc,dbp1,sbp1loc,sbp1)
        for mF = 1:nF
            xF =
find(dbpFloc<sbpFloc(mF));
            if isempty(xF) || ...
               sbpFloc(mF)-
dbpFloc(max(xF)) > 100      % "will
need change"
                    bppairsF(1,mF) =
0;
                    bppairsF(2,mF) =
0;
                    bppairsF(3,mF) =
0;
                    bppairsF(4,mF) =
0;
            else
                    bppairsF(1,mF) =
dbpFloc(max(xF));
                    bppairsF(2,mF) =
dbpF(max(xF));
                    bppairsF(3,mF) =
sbpFloc(mF);
```

```
                    bppairsF(4,mF) =
sbpF(mF);
            end
        end
bppairsF = bppairsF'; % now becomes a
column vectors
bppairsF(dbpFloc,dbpF,sbpFloc,sbpF)
bppairsF(~any(bppairsF,2),:) = [];
```

Find the mean

```
qF = size(bppairsF,1)-1;
qtF = size(bppairsF,1);
MAP_F = zeros(1,qF);
tMAP_F = zeros(1,qtF);
for pF = 1:qF
    AUC_wF =
trapz(fullFdat(bppairsF(pF,1):bppairs
F(pF+1,1)));
    wF_time = bppairsF(pF+1,1)-
bppairsF(pF,1);
    MAP_F(pF) = AUC_wF/wF_time;
end
MAP_F = MAP_F';
for ptF = 1:qtF
     tMAP_F(ptF) =
fix(mean([bppairsF(ptF,1)
bppairsF(ptF,3)]));
end
tMAP_F = tMAP_F';
```

Add MAP_F to bppairsF

for that equalize the dimensinons of the MAP_F and the bppairsF

```
nMAP_F = zeros(size(bppairsF,1),1);
nMAP_F(1:size(MAP_F,1),
1:size(MAP_F,2)) = MAP_F;
% CHECK can be done by -->
isequal(size(nMAP_F,1),
size(bppairsF,1))
nbppairsF_nMAP_F = cat(2, bppairsF,
tMAP_F, nMAP_F);
```

Find the Point of intervention

To find the common point of intervention to calculate the time to death and compare. first find the time location of the MAP_F where the MAP drops below a certain set percentage (P_cent)

```
% mean of 1st 100 values to get base
line
Mean1st100 =
mean(nbppairsF_nMAP_F(1:100,6)); %
gives me the initial baseline value
P_cent = .5;
dropMAP = Mean1st100-
(P_cent*Mean1st100);
[RoD, ~] =
find((1>abs(nbppairsF_nMAP_F(:,6)-
dropMAP)) & ...
    (abs(nbppairsF_nMAP_F(:,6)-
dropMAP)>0),3600000,'first');
                                %
'190214_WR_SS' needs second drop
[RoD0, ~] =
find(RoD(:,1)>10000,1,'first');
RoD1 = RoD(RoD0,1);

% Find the time  of drop in MAP.. i.e
the Location in the whole
TD1 = nbppairsF_nMAP_F(RoD1,5);
% [vLTD1, LTD1] =
min(abs(nbppairsF_nMAP_F(:,5)-TD1));
these two lines are redundant!!! can
remove them after checking
% LTD_F1 = nbppairsF_nMAP_F(LTD1,5);
% approx  <<<-----
```

Make the selection on the graph

First find the location of the comment(s) in the data. If there are more than one segments in the recording the values (location) of the comments will change when the whole data is taken together as the location of each segment starts from zero.

```
if size(datastart,2) > 1
    ncom = zeros(size(com,1),2);
      for c = 1:size(com,1)
         if com(c,2) == 1
             ncom(c,1) = com(c,3);
          elseif com(c,2) > 1
           ncom(c,1) =
com(c,3)+dataend(com(c,2)+com(c,1));
         end
       end
  ncom(:,2) = com(:,5);

  Xec =
contains(cellstr(comtext),'EColi'); %
find text 'EColi' is present or not
    locEC = find(Xec);          %
find the non-zero location
    Xec1 = ismember(ncom,locEC);
    locEC1 = find(Xec1)-size(ncom,1);
    SelecEC = ncom(locEC1,1);

    % For any other comment
 end

if size(datastart,2) == 1
    Xec =
contains(cellstr(comtext),'EColi'); %
find the location of text 'EColi'
    locEC = find(Xec);
    SelecEC = com(locEC,3);
end
```

Find the location of the comment EColi in the compiled nbppairsF_nMAP_F data.

```
[~,nSelecEC] =
min(abs(nbppairsF_nMAP_F(:,5)-
SelecEC));% Find SelecEC in
nbppairsF_nMAP_F
%{%}
```

To the time taken for different events:

```
% Time to drop (first drop value) is
(in hours); 3600000 milliseconds is 1
hour
TTD1 = (TD1-SelecEC)/3600000;
%Location of death of the animal
loDeath = size(nbppairsF_nMAP_F,1);
% duration for the death of the
animal
lTDe = nbppairsF_nMAP_F(end,5) - TD1;
% lTDe - length of Time to Death
TDe = lTDe/60000; % in minutes

% Find the max and min values on the
Y axis
maxMap = max(nbppairsF_nMAP_F(:,6));
mM = 900:-100:100;
MM = find((mM-maxMap)>0 & (mM-
maxMap)<100);
yVal = mM(MM);

%FInd the X-axis tick values and
labels
nR = (size(nbppairsF_nMAP_F,1));
%no of rows
nR1 = num2str(nR,3);        %convert
rowvalue into structure, precision
value is 1
nR2 = str2double(nR1(end));
nR3 = ceil(str2double(nR1(1:4)));
nR4 = nR+1000;
tR =
abs(mean(nbppairsF_nMAP_F(end,[1,3,5]
)));  %time reresented in last row
(ms) total time
tR1 = (tR/nR)*nR4; %Time value
corresponding to each X axiz value
tR2 = tR1/3600000; % the time
represented in X axis converted to
Hours
xt1 = tR2/10; % ten divisions of the
time (tick value of time in hours)
xt2 = round(0:xt1:tR2, 2); % the
whole set of time tick values
xt3 = round(nR4/10, 1,'decimals');
```

Visualization

```
mygreen = [0 0.6 0.2];
myblue = [0 0.45 0.75];
myred = [0.85 0.33 0.1];
mybrown = [0.64 0.08 0.18];
dotsize = 25;
                                % plot
Selec1Data
SF = size(fullFdat,2);
MS =
milliseconds(nbppairsF_nMAP_F(:,5));
figure('Visible','on')
plot(nbppairsF_nMAP_F(:,6),'linewidth
',1,'color',myblue);
hold on
xlabel('Time (hr)');
ylabel('Pressure (mmHg)');
xlim([0 nR4])
ylim([0 yVal])
xticks(0:xt3:nR4)
xticklabels(xt2)
mystdaxis(gca)
annotation('textbox',[0.1841220238095
23 0.748217966645743
0.0957142875705447
0.0638095247631981],...

'String',"EColi",'FitBoxToText','on')
;
line ([nSelecEC nSelecEC], [0
yVal],'LineStyle',
':','LineWidth',1.5,...
    'Color',[0.8 0.12653
0.52365],'DisplayName' ,"EColi")
annotation('textbox',[0.6231290035049
53,0.573173629157063,0.08375634692243
7,0.065429688477889],...
    'String',"50%
drop",'FitBoxToText','on');
line ([RoD1 RoD1], [0
yVal],'LineStyle','-.'
,'LineWidth',1,...
    'Color',[0.6650 0.365
0.481],'DisplayName' ,"Drop in MAP")
annotation('textbox',...

[0.827275954798158,0.617733891935384,
0.060913706783918,0.065429688477889],
...

'String',"Death",'FitBoxToText','on')
;
line ([loDeath loDeath], [0
yVal],'LineStyle','--',
'LineWidth',1.5,...
    'Color',[0.64 0.08
0.18],'DisplayName' ,"Death")
legend('MAP', 'EColi', 'MAP
Drop','Death')
annotation('textbox',...
    [0.349138148161029
0.687142857142857 0.342647566124685
0.192609380597422],...
    'String',sprintf('MAP Drop_{50} =
%d/mmHg\nDrop Time_{Hr} = %.1f
```

```
Hours\nDeath Time_{Hr} = %.1f
minutes',...
    fix(dropMAP), TTD1,
TDe),'FitBoxToText','on',...

'HorizontalAlignment','left',...
        'FontName','Georgia',...
        'FontSize',9,...
        'FontAngle','italic',...
        'FontWeight','normal')
title([FF_name,' Complete
pic'],'Interpreter','none');
mystdpng_ls([FF_name,' Complete
pic'])
```

Saving the required variables

```
ReqPar_F = {'Initial MAP',Mean1st100;
...
    sprintf('%0.0f percent drop in
MAP',P_cent*100),dropMAP;...
    'Time to MAP drop',TTD1;'Time to
death',TDe};
xlswrite(sprintf('%s.xlsx',FF_name),R
eqPar_F,1,'F1');
save(sprintf('%s_clean.mat',FF_name),
'bppairsF','dbpF','sbpF',...

'Mean1st100','dropMAP','nSelecEC','nb
ppairsF_nMAP_F','nMAP_F',...
    'TTD1','TDe', '-append');
% clearvars
```