RABBIT MODEL FOR SEPTIC SHOCK USING BACTERIAL STRAIN ISOLATED FROM PATIENTS WITH SEPSIS

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

Department of Physiology
Christian Medical College
Vellore

May 2020

Department of Physiology
Christian Medical College
Bagayam
Vellore 632002
Tamil Nadu

Phone: +91 416 228 4268
Fax: +91 416 226 2788,
226 2268
Email:
physio@cmcvellore.ac.in

CERTIFICATE

This is to certify that this dissertation work titled **Rabbit model for septic shock using bacterial strain isolated from patient with sepsis** is a bona fide, original work of the candidate **Dr. Aravindhan V.** with registration number **201715351**, 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.

Dr. Elizabeth Tharion
Professor & Head
Department of Physiology
Christian Medical College
Vellore – 632002

Dr. Sathya Subramani
Professor and Guide
Department of Physiology
Christian Medical College
Vellore-632002

DECLARATION

I hereby declare that the investigations that form the subject matter of this dissertation titled **Rabbit model for septic shock using bacterial strain isolated from patient with sepsis**, 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.

Dr. Aravindhan V.
Department of Physiology
Christian Medical College
Vellore-632002

Department of Physiology
Christian Medical College
Bagayam
Vellore 632002
Tamil Nadu

Phone: +91 416 228 4268
Fax: +91 416 226 2788,
226 2268
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Dr. Anna B Pulimood
Principal
Christian Medical College
Vellore – 632002

Department of Physiology
Christian Medical College
Bagayam
Vellore 632002
Tamil Nadu

Phone: +91 416 228 4268
Fax: +91 416 226 2788,
226 2268
Email:
physio@cmcvellore.ac.in

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Dr. Sathya Subramani
Professor and Guide
Department of Physiology
Christian Medical College
Vellore – 632002

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ABSTRACT

BACKGROUND

Septic shock is the leading cause of non-coronary deaths in ICU. Not only in adults in neonates has it still remained the dominant killer. Still the exact pathophysiology of sepsis related death is not fully explored.

AIM

To create an animal model of septic shock in rabbits using bacterial strains isolated from patients.

OBJECTIVES

(i) To standardize the rabbit model of septic shock by injecting the correct concentration of bacterial strains isolated from patients.

(ii) To study the effect of Nor Adrenaline in the above septic shock induced rabbit.

(iii) To study the effect of Phentolamine in the above septic shock induced rabbit.

METHOD

New Zealand white rabbits (NWR) were randomly selected from animal house with weight of around 2.5–3 kgs .The animals were anaesthetized with intramuscular injection of ketamine (35 mg per kg body weight) and xylazine (5 mg per kg body weight). ECG leads and respiratory belt were placed and connected to the CMC data acquisition system. The dorsal aspect of the ears were shaved, draped, painted and intravenous catheter was placed in situ. Maintenance dose of anaesthesia (ketamine 4ml+midazolam 6ml) was infused along with normal saline for fluid replacement at the rate of 4 ml/kg/hr. Central ear artery was cannulated and connected to a pressure transducer. The animals were observed for stabilization of the above vital parameters. Escherichia coli strain isolated from patients of septicaemia was reconstituted in 2ml of normal saline and injected intravenously into the rabbit. Changes in ECG wave rate, morphology and respiratory rate (increase/decrease) was noted. Intra-arterial blood pressure was monitored.

(i) In the control group, a drop in blood pressure was expected due to onset of septic shock. The animals were monitored till death. Time duration,

- From injecting the bacterial culture to onset of hypotension was noted.
- From onset of hypotension to death was noted.

(ii) In another group of animals, after the onset of hypotension, Nor Adrenaline was administered intravenously and the time duration to death was noted.

(iii) In another group of animals immediately after injecting the bacterial strain Phentolamine was given intravenously, and the time duration to death was noted.

RESULTS

- Animals that received *E.coli* alone died within 136±22.19 minutes
- Animals that received *E.coli* + NA - time to death was prolonged 354±58.67 minutes
- Animals that received *E Coli*+ Phentolamine died earlier in 49.75±26.86 minutes
- There was a combined Metabolic and Respiratory acidosis after *E.coli*. The metabolic acidosis was associated with significant Hyperchloremia, Anion Gap did not show a significant difference. Significant Hypernatremia was seen.
- Mean Arterial Pressure increased in the group that received *E Coli* + Noradrenaline compared to the group that received *E Coli* + Phentolamine

CONCLUSION

1. Effective Rabbit model of Escherichia *Coli* septic shock is developed to study the pathophysiology of septic shock related deaths.
2. Response to known intervention were as expected
    a. Alpha agonist increases MAP thus delaying death in septic shock.
    b. Alpha antagonist along with the additive effect of Escherichia Coli hastened death in septic shock.
3. The model can be extended using other live strains of bacteria.

Keywords – Septic shock, *Escherichia Coli*, Alpha agonist, Alpha antagonist

1. INTRODUCTION

Sepsis is one of the most aged puzzling syndromes in the medical field and it is still potentially a major public health concern even in well developed countries.

Historically sepsis where considered as a condition where the human tissue decays generating foul smell leading to wound rotting. Some opined sepsis as a creditable event that promotes wound healing whereas a few proposed sepsis as blood poisoning where the organism enters the host and spread through blood to all the parts of the body based on germ cell theory. But with the recent advances in the medicine even after elimination of the causative organism from the host the germ theory fails as the mortality still remains high despite early and aggressive therapy. Astonishingly we are still wondering what exactly sepsis related shock does to the physiology of the human beings.

2. AIM

To create an animal model of septic shock in rabbits using bacterial strains isolated from patients.

3. OBJECTIVES

(i) To standardize the rabbit model of septic shock by injecting the correct concentration of bacterial strains isolated from patients.

(ii) To study the effect of Nor Adrenaline in the above septic shock induced rabbit.

(iii) To study the effect of Phentolamine in the above septic shock induced rabbit.

4. REVIEW OF LITERATURE

Septic shock is one of the leading cause of non-coronary deaths in intensive care patients (1). Not only in adults but in neonates ,sepsis is still the dominant killer that is in a constant threat (2). Sepsis related death is a major concern to the mankind not only in terms of mortality, morbidity but also it is a huge threat in financial aids as it warrants stay in a tertiary care center for a prolonged period of time until recovery. Mortality rate exceeds 50%, due to vasodilation leading to hypotension, which progressively worsens despite hemodynamic support (3).

Even in developed countries the statistics from the World Health Organization (WHO) points out that at least 50% of the children less than four years admitted in Intensive Care Unit die due to sepsis or septic related shock (6), (7),(8). The outcome of sepsis related mortality is also dependent on multiple factors like accessibility and affordability towards the health care especially in pediatric population (4).

It is very difficult to diagnose septic shock earlier in these patients because of the presence of multiple comorbidities they present with(5). Deregulated response of the host immune system to uncontrolled infection leads to severe sepsis which progresses to multiple organ dysfunction and finally death (6).

4.1. EPIDEMIOLOGY

Western countries data related to sepsis related mortality may vary with origin of data base, collection, diagnosis, retrospective review and prospective observational studies(7),(8),(9). Globally approximately case fatality rate due to sepsis is 30%, severe sepsis 50%, septic shock 80% even in developed countries, the rate may shoot up even higher in developing countries and may be drastic in underdeveloped countries (10).

Latest studies in India shows that the death toll among the patients admitted in Intensive Care Unit is almost 56%, in hospital 63% and post 28 days 63%. Overall 34% of patients admitted with sepsis die annually in India (11).

Cost of medical care for sepsis related admissions in United States alone is 16.7 billion US dollars per year (12). Unfortunately sparse studies are being done on sepsis in India which leads to inadequate data. Even those sparse studies mainly concentrates on the antibiotic usage, microbiological profile, resistance pattern, outcome from illness rather than the epidemiology per se(13),(14).

There is an alarming rise of septic shock as there are more resistant microorganisms present in the environment, aggressive surgeries performed by the health care professional and most importantly immunosuppression either due to diseases or usage of immunosuppressive drugs.

4.2. DEFINITIONS

The analogue for sepsis keeps updating every time due to recent advances in the field of medical science and better understanding of the pathophysiological mechanism they bring in every individual patient.

Systemic inflammatory response syndrome (SIRS) can be the referred term for *sepsis* (15) which ideally should have at least two of the four clinical signs given below

4. Increase in body temperature (more than 38°C)
5. Increase in heart rate (more than 100 bpm)
6. Increase in respiratory rate (> 20 per minute)
7. Extreme deviation of White blood cells count (> 12,000 cells/cu.mm or < 4000 cells/cu.mm)

Sepsis can be considered as a sub class of SIRS provided that clinically infection must be documented in the affected individual(15). Severe form of SIRS may progress to *Multiple Organ Dysfunction Syndrome (MODS)* which can be either

- Direct eg., Mendelson's syndrome in which there is aspiration of the stomach contents into the lungs causing chemical pneumonitis
- Indirect eg., Traumatic injury to a particular organ can lead to a widespread inflammatory response to other organ system as well.

Severe sepsis is when there is dysfunction of multiple organ systems. Onset of multiple organ failure may be unique for each patient and the course may vary within the same

individual as well(15). The immune response of the host against the invading pathogen can lead to a varying degree of organ hypofunction during severe sepsis.

A. *Cardiovascular*: Reduction in the arterial systolic blood pressure <90 mmHg or MAP <70 mmHg which react to the administration of intra venous fluids.
B. *Respiratory*: If lung is the only hypo functioning organ $PaO_2/FiO_2 \leq 200$ or $PaO_2/FiO_2 \leq 250$
C. *Renal*: Reduction in the urine output <0.5mL/Kg/ hour despite administering adequate fluid resuscitation
D. *Hematologic*: 50% decrease in the platelet count from the highest value that is recorded in previous 72 hours
E. *Unexplained metabolic acidosis*

Bacteremia can be defined as the growth of bacteria when the blood is cultured, infection need not be proven for diagnosing sepsis at onset(15).

The term *septic shock* is reserved for severe sepsis that induces hypotension (systolic BP <90 mm Hg or decrease of >40 mm Hg from the baseline) which is not responsive to fluid administration and may warrant the use of vasopressor agents(16).

Refractory septic shock is one which does not respond to both IV fluids and high-dose vasopressor therapy (17).

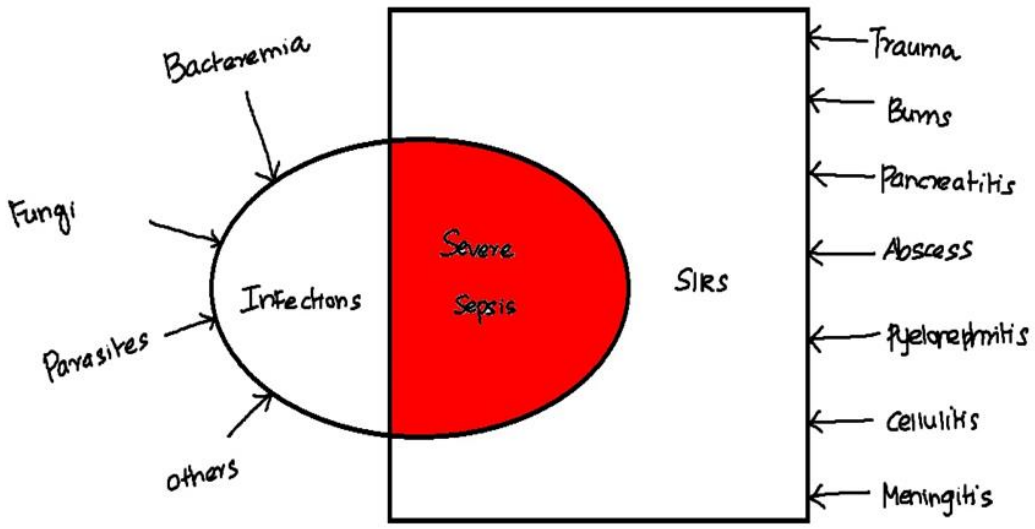


Figure 1. Definition and causes for sepsis

4.3. CAUSATIVE AGENTS

Sepsis can be caused by gram positive (18), gram negative bacteria (19), fungi and rarely protozoa. The approximate distribution of proportion among the causative agents is as follows:

I.	Gram positive bacteria	-	50%
II.	Gram negative bacteria	-	40%
III.	Anaerobic organisms	-	2%
IV.	Fungi	-	5%
V.	Others	-	3%

The mechanism in which these organisms cause sepsis may vary from each other (20). Commonest organisms in each categories are tabulated below:

Gram positive bacteria	**Gram negative bacteria**	**Fungi**
Staphylococcus aureus	Pseudomonas aeruginosa	Candida albicans
Coagulase-negative Staphylococcus	Escherichia coli	
Streptococcus pyogenes	Neisseria meningitidis	
Streptococcus pneumoniae	Serratia	
Enterococci	Proteus	
	Klebsiella pneumoniae	

Table 1.showing the causative organisms for sepsis

The frequent sites of origin of infection leading to septic shock are abscess (especially intra-abdominal), primary bacteremia, pneumonia, pyelonephritis, peritonitis, cellulitis, cholangitis, meningitis and necrotizing fasciitis. One should not neglect the fact that Nosocomial infections or hospital acquired infections are also most common leading to the above mentioned causes of sepsis.

4.4. ESCHERICHIA COLI

The intestinal system of human beings predominantly contains non-acid fast, non-sporing aerobic Gram negative bacilli, grouped under the family Enterobacteriaceae.

Majority of the intestinal commensal bacilli are lactose fermenters, most common among them being *Escherichia coli* or coliform bacilli (21),(22). Escherich was the first one to describe the colonic bacilli using the name *Bacterium coli commune* in the early 80's. Several species are grouped under the family enterobacteriaceae like *E.fergusonii, E.hermanii, E.vulneris* and *E.blattae*(23),(24),(25),(26). Of the above mentioned species *E.blattae* has been isolated in the gut of cockroaches (27), others being frequently isolated from clinical samples. In contrary to other species among the family *Escherichia Coli* is the only parasite seen in both animal and human intestine. When shed through feces they remain viable only for a particular number of days. Hence *E.coli* when detected in the drinking water sample can be a clue suggesting recent pollution with animal or human feces (28),(29).

4.5. MORPHOLOGY OF *ESCHERICHIA COLI*

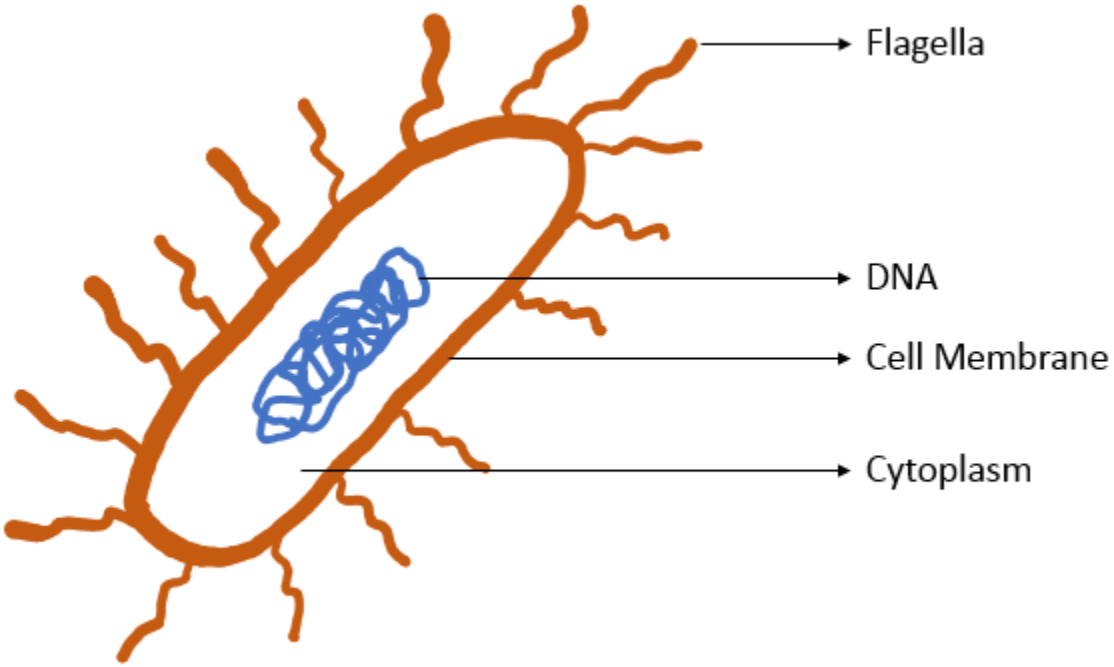
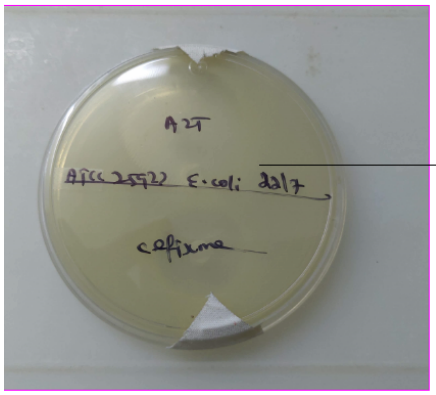


Figure 2. Morphology of *Escherichia Coli*

- Rod shaped
- Motility by peritrichate flagella
- Arranged either singly or in pairs
- Fimbriae and Poly saccharide Capsules found in some strains

4.6. CULTURE CHARACTERISTICS



Freshly isolated *Eshericha Coli* sample in a petri dish

Figure 3. Freshly isolated culture of *Escherichia Coli*

The colonies of these bacteria's are thick, moist, large, whitish grey, smoothly opaque or translucent. There are two forms of the colonies

a. Smooth form (S) which are seen in freshly isolated sample and is easily emulsifiable in normal saline (30).
b. Rough form (R) which is a product of repeated sub cultures has irregular surface and is often auto agglutinable in normal saline (30).

In a nutrient broth the growth of these strains occurs as a turbidity with heavy deposits which disperses with vigorous shaking.

4.7. ANTIGENIC STRUCTURE

Serotyping of *Escherichia Coli* is mainly based on the three antigens(31),(32),(33)

1. Capsular K antigen
2. Somatic O antigen
3. Flagellar H antigen

K antigen predominantly present in the envelope (34), which encloses O antigen thus contributing to the virulence of the coliform bacilli. Strains that are found in the intestinal flora usually do not possess K antigen, whereas normal colonic strains has early part of O antigen and pathogenic strains has later part of O antigen.

4.8. VIRULENCE FACTORS

The virulence of Escherichia Coli is mainly by the surface antigen, toxins produced by them and partly by the fimbriae (35). The lipopolysaccharide surface O antigen has endo toxic activity (36) which protects the organism from bactericidal effect of the Complement system and phagocytosis.

Plasmid coded fimbriae which is not chromosomally determined enumerate on to the virulence especially in urinary tract infections where the P fimbriae has increased affinity for the uro epithelial cells (37).

Toxins produced by Escherichia Coli are of two types

a. Hemolysin
b. Enterotoxin

Hemolysin though not related to pathogenesis is mostly produced (38).

Enterotoxin generally causes diarrhea in human has three distinct subtypes

1. Heat labile toxin (LT)

   Discovered in 1970 by rabbit ileal loop method by injecting the freshly isolated *Escherichia Coli* into the ileum of the rabbit (39) which produced increased outpouring of the fluid in the intestine resulting in the ballooning of the ileum.

   LT has two sub units A and B. Sub unit B binds to Gm-1 ganglioside receptor on the intestinal cells which activates A (40). Subunit A when activated leads to the formation of A_1 and A_2. A_1 in turn activates adenylyl cyclase to produce cAMP leading to outpouring of water and electrolytes into the gut lumen thus causing diarrhea (41),(42). Mechanism of causing diarrhea where similar to cholera toxin (43).

2. Heat stable toxin (ST)

   Heat stable toxins discovered in 1970 are weakly antigenic which has two sub units.

   ST_A activates cGMP in the intestinal cells leading to fluid accumulation in the intestine of infant mice (44), whereas ST_B does the same in piglet intestine mechanism of which is not conclusive (45).

3. Verotoxin (VT)

   Also known as Shiga like toxin (46) were discovered in 1977 due to its cell destroying effect on the Vero cell lines derived from African green monkey

kidney cells (47). VT exhibited enterotoxicity in rabbit ileal loop study similar to heat labile toxins (46).

4.9. CLINICAL INFECTIONS

The four main clinical syndromes caused by Escherichia Coli are as follows

1. Urinary tract infections
2. Diarrhea
3. Pyogenic infections
4. Septicemia

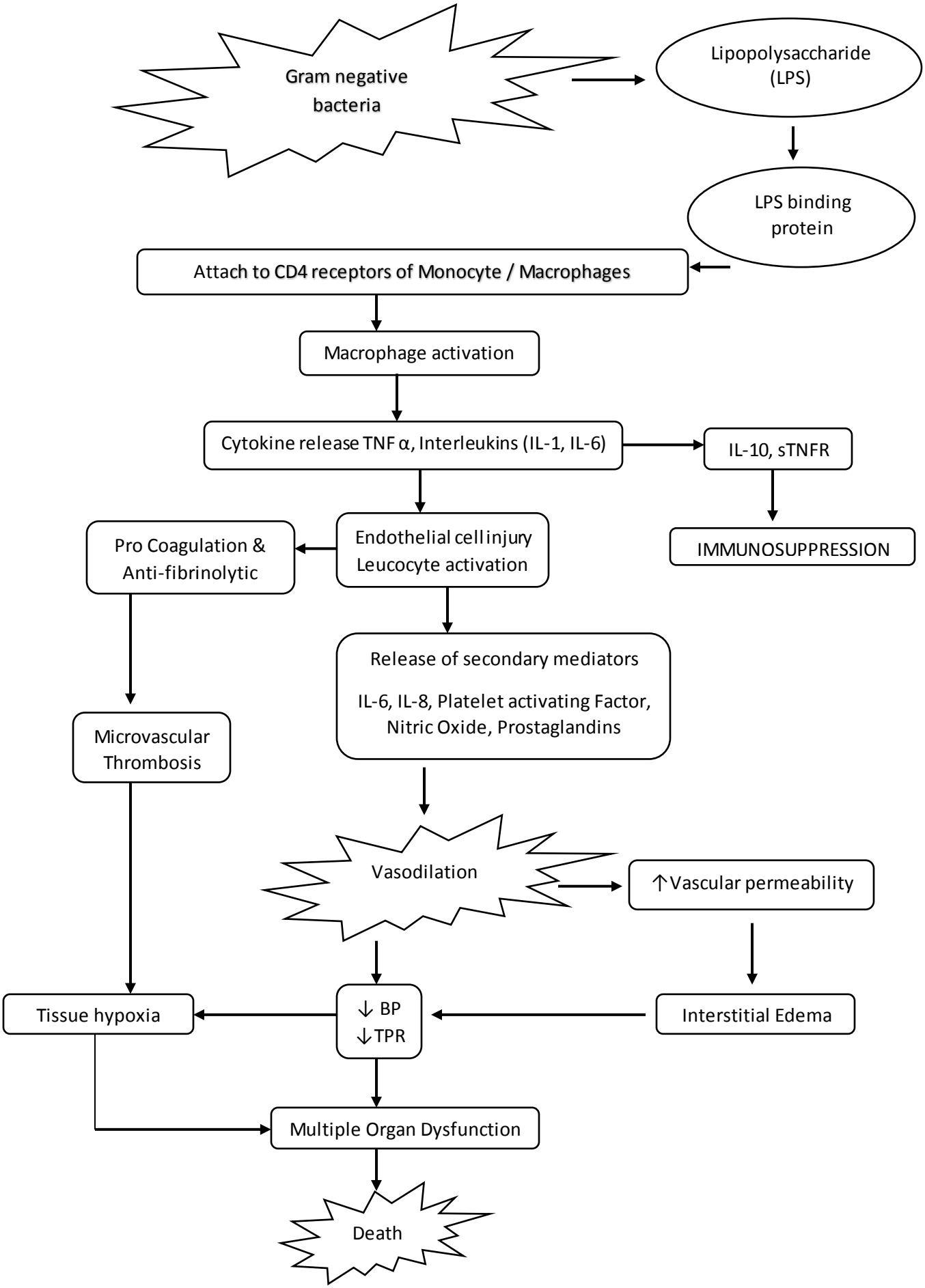
4.10. PATHOPHYSIOLOGY

Septic shock first triggers inflammatory process which involves a sequence of events, which is later followed by anti-inflammatory events and finally immunosuppression contributing to death. The process involves a vicious cycle of pro inflammation and pro coagulation resulting almost like a positive feedback phenomenon.

The cell wall components of the bacteria like lipopolysaccharide (LPS) possess receptors for mononuclear inflammatory cells, neutrophils, endothelial cells which in turn leads to cell activation. A special type of receptor, Toll-like receptor (TLR) (48) spots the bacterial cell wall components and leads to septic cascade (49),(50). Certain bacterial proteins as well as Nucleotide oligomerization domain protein (NOD 1&2)

are also identifies by G protein coupled receptors. When the inflammatory cells are activated they release various inflammatory mediators like Interleukins (IL-1, IL- 12, IL-18), Interferon γ, High mobility group box 1 protein (51). Platelet activating factors ,Reactive oxygen species , prostaglandins are also produced which either behave like a pro coagulant or activates the complement activating cascade (52). Damaged endothelial cells and the inflammatory cytokines produced activation of leukocytes further leads to the release of secondary mediators of inflammation which enhances vasodilation, increased vascular permeability, interstitial edema (49).

The inflammatory cytokines leads to insulin resistance by destroying the glucose transporter's in skeletal and cardiac muscle eventually producing hyperglycemic state. The increased blood glucose levels blunts the neutrophil function thus the bactericidal effect (53). The cytokine like mediators also activates secondary anti-inflammatory mediators which abolishes the immune system finally shutting down the entire immune support (50),(49),(52). All the events in the septic shock finally causes tissue ischemia , decreased perfusion, immunosuppression leading to multiple organ damage finally culminating to death.



Flowchart 1. Pathophysiology of septic shock

4.11. RISK FACTORS

The efficient risk factors in an individual that can cause sepsis and septic shock are

i. Extremes of ages (pediatric & geriatric population)
ii. Rapid increase in the number of drug resistant micro organisms
iii. Immunocompromised states
iv. Increase in the usage of cytotoxic, immunosuppressant drugs
v. Aggressive invasive management
vi. Severe malnutrition
vii. Malignancy
viii. Acquired immune deficiency syndromes
ix. Upsurge sensitivity in diagnosis
x. Increase in organ transplantation
xi. Increased awareness in medical professionals

Any factor that is likely to reduce the immune status of an individual can lead to the development of sepsis and septic shock(54),(55). Malnutrition alone can increase the chances of infections like bacteremia, tuberculosis, enteric gram negative bacteremia, viral hepatitis and fungal infections. Malnutrition when accompanied by vitamin deficiencies can cripple the function of WBC's which plays an important role in host defense mechanism(55).

4.12. BIOMARKERS FOR PREDICTION OF MORTALITY AFTER SEPTIC SHOCK

PIRO system of classification can be used to predict the mortality after septic shock. Only a few studies have validated this system of prediction. In this system P stands for Prediction, I for insult or injury, R for response and O for Organ dysfunction (56),(57).

	CLINICAL	LABORATORY
Prediction (P)	Gender Age Immunosuppression Alcohol	Hereditary factors
Insult / Injury (I)	Source of primary infection	Causative organism
Response (R)	Blood pressure Heart rate Respiratory rate Temperature	TLC C- reactive protein
Organ dysfunction (O)	GCS Urine output MAP	Serum Creatinine LFT CBC Coagulation profile

Table 2. PIRO system for septic shock

4.13. CLINICAL MANIFESTATIONS

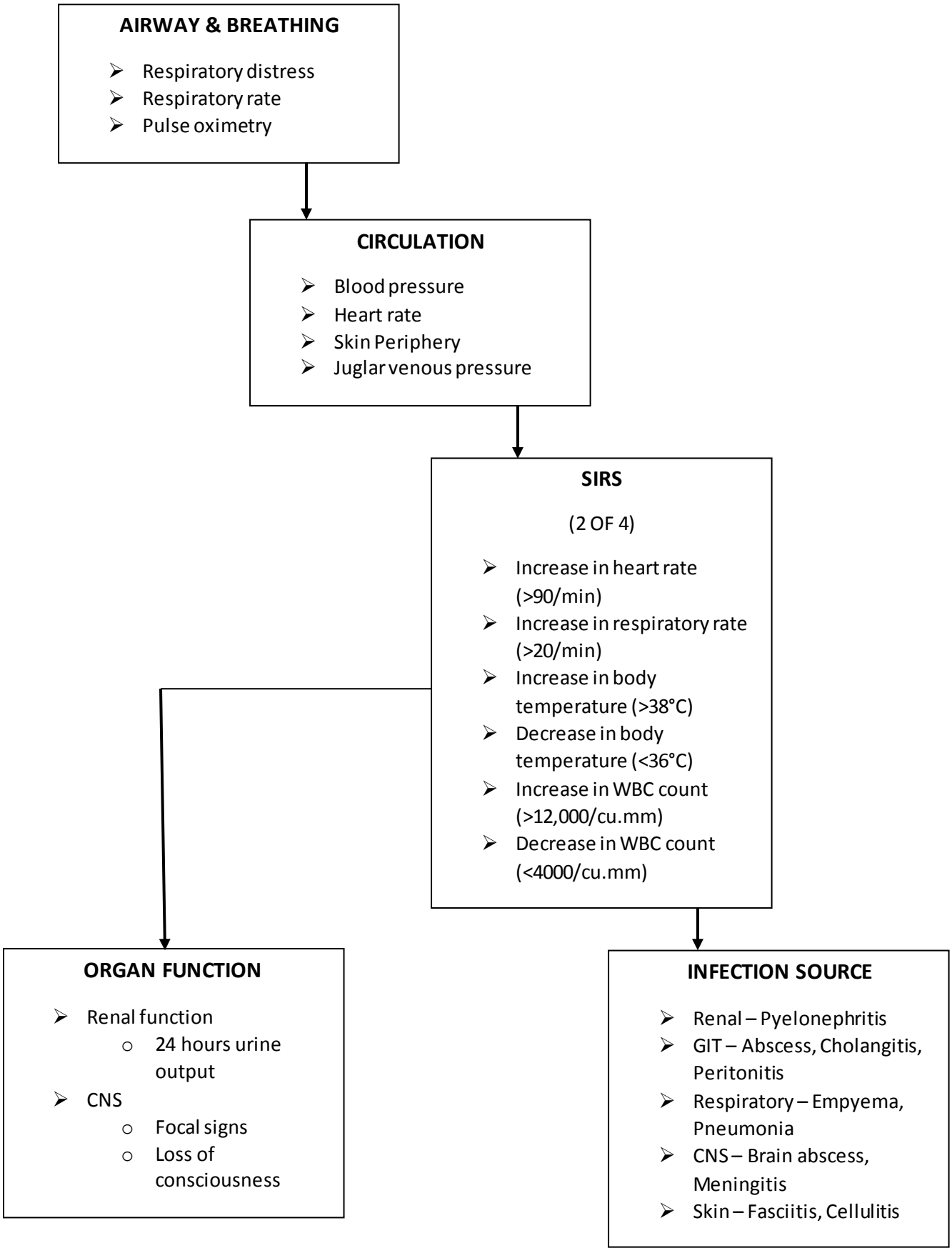
The clinical manifestations in sepsis may vary from patient to patient depending upon the site, severity, source of the infection, characteristics of the host and most importantly the time course of sepsis before diagnosis and intervention. These manifestations are complicated as they are often super imposed on the signs and symptoms of the underlying infection (16).

Organ dysfunction leads to

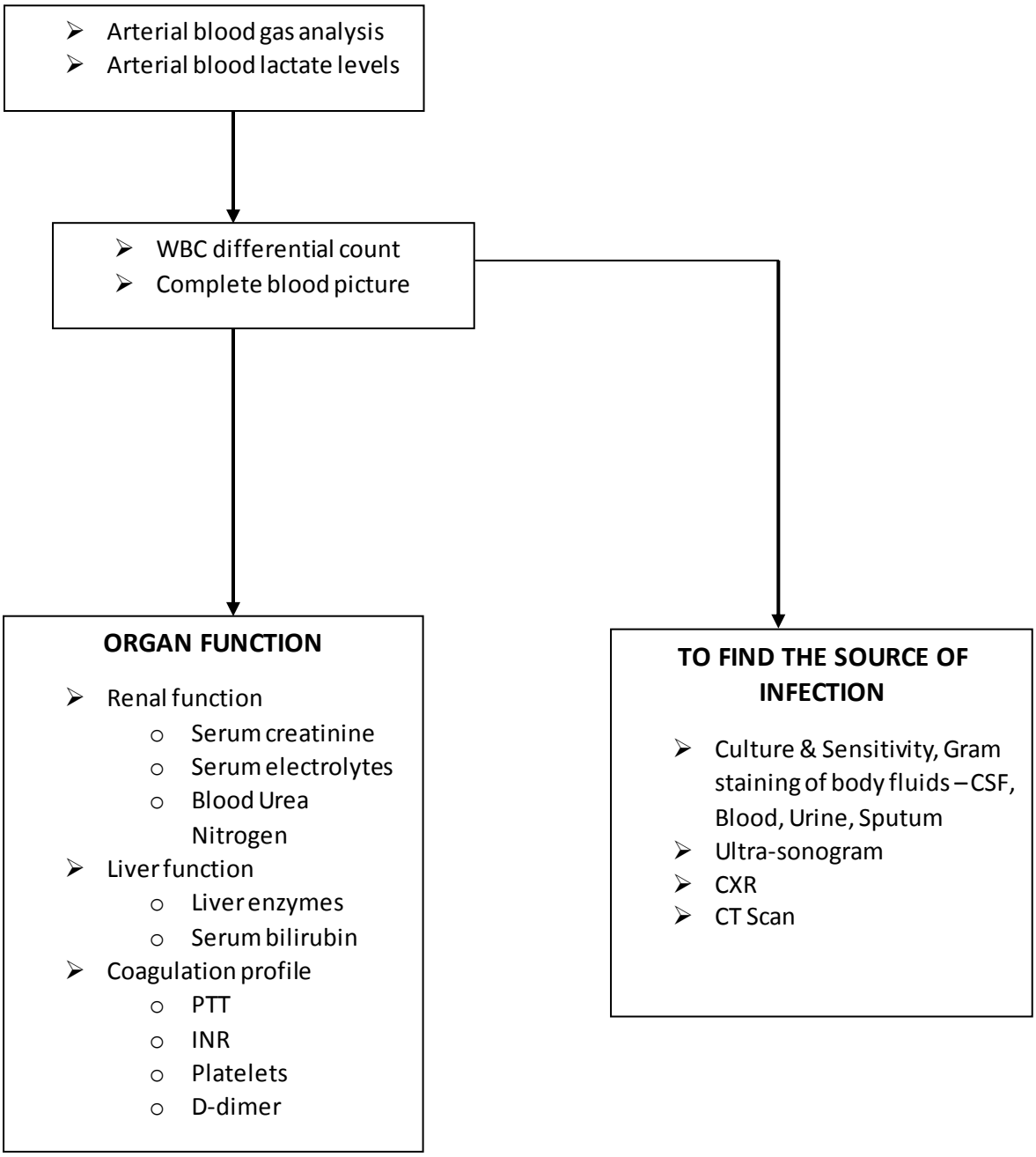
- Hypotension
- Disseminated intravascular coagulation
- Disorientation
- Confusion
- Altered mental status
- Hepatic & canaliculi dysfunction,
- Adrenal dysfunction
- Cutaneous lesions
- Nausea
- Vomiting
- Ileus
- Diarrhea
- Respiratory distress (58).

The cumulative organ hypo function determine the outcome as well as the prognosis, even after survival immunosuppression may pave way for secondary infections (59).

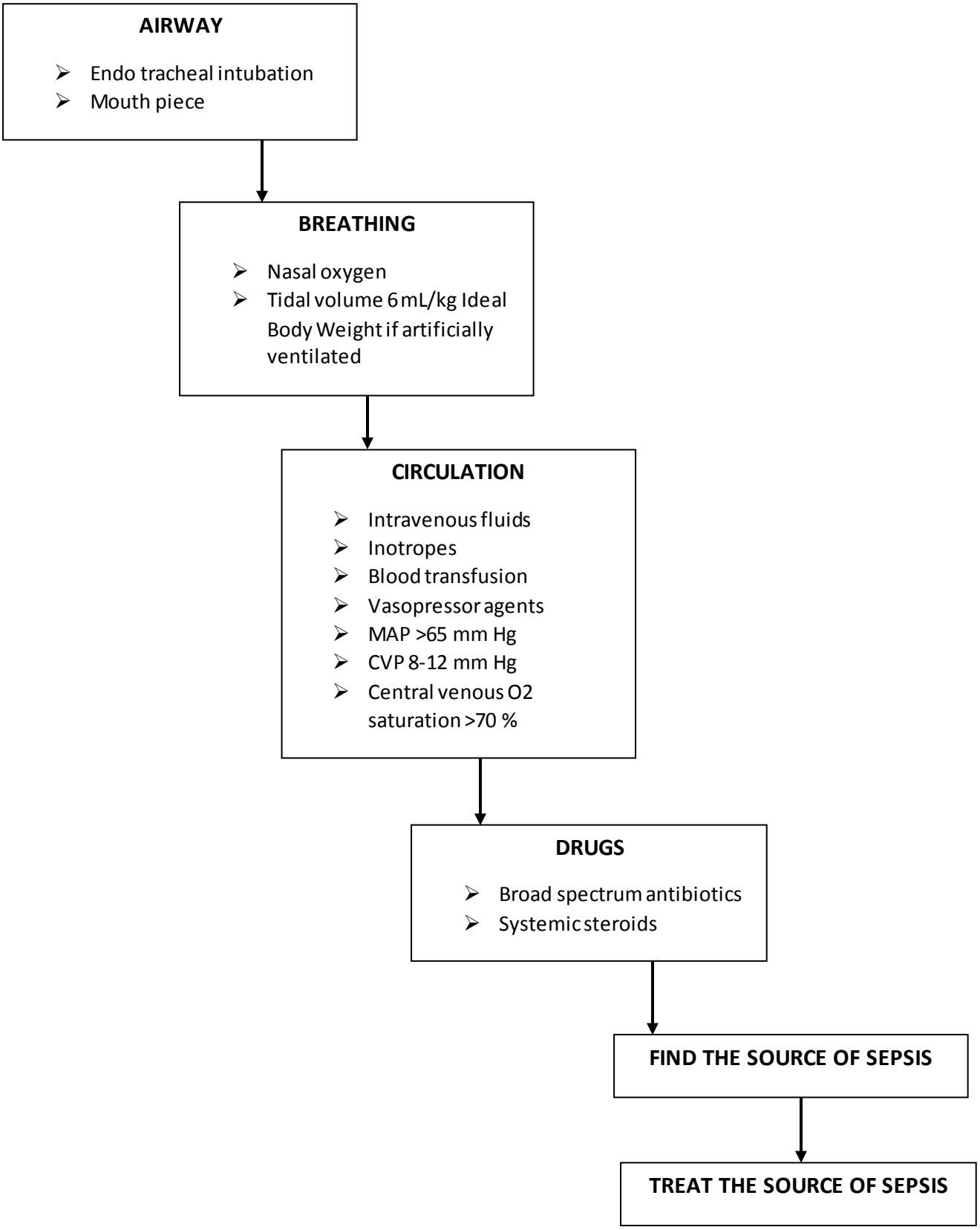
4.14. FLOWCHART FOR CLINICAL EVALUATION OF SEPTIC SHOCK



4.15. FLOWCHART FOR LABORATORY EVALUATION OF SEPTIC SHOCK



4.16. FLOWCHART FOR MANAGEMENT OF SEPTIC SHOCK



4.17. ANIMAL STUDIES ON SEPSIS AND SEPTIC SHOCK

Animal studies provides a platform for developing newer treatment strategies and it is an important link between successful *in vitro* and safer human use. The designing of the medical research project should be in such a way that minimum number of animals are required provided there is an established standards of animal care or when no other alternative is available (60). Key principles of **3 R's** in using animals for research are as follows

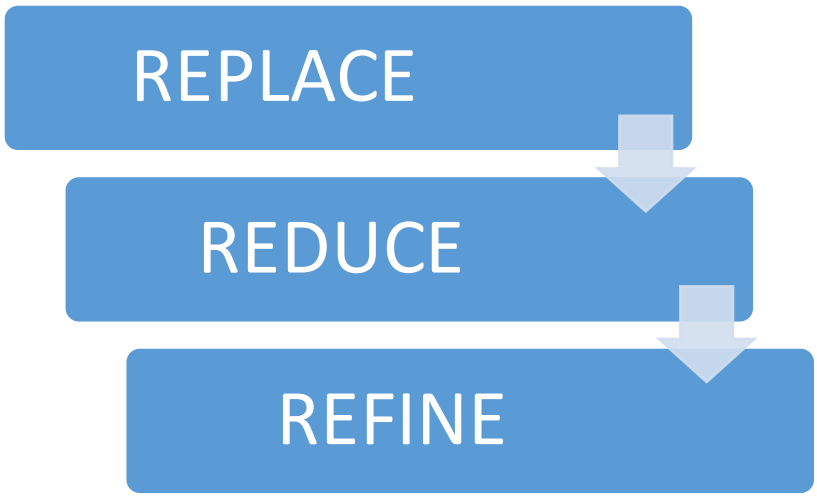


Figure 4. *3 R's* in animal research

Replace – work without animals whenever possible

Reduce – when you cannot avoid use animals, use the less possible

Refine – Using few animals with the most respect for the animal

4.18. SELECTION OF ANIMAL MODEL

The animal suitable for the medical research must have an anatomy and physiology similar to that of human beings with a parallel pathophysiology of the disease process. Clinical situation in these animal models should mimic human clinical status (60).

Commonly available animal models for research purpose are Rat, Sheep, Mice, Pig, Rabbit, Cat, Goat and primates. Of these animal models Rabbit is best suited for studying tissue engineering and in surgical fields.

4.19. ANIMAL RESEARCH MODEL IN INDIAN SCENARIO

In India most of the animals are considered sacred on religious backgrounds. Further hurdles are poor infrastructure, lack of expertise in handling animals, inadequate funding and finally lack of interest among the medicos.

The Government of India has approved the usage of animals for research purpose which follows the guidelines laid down by Indian National Science Academy. The guidelines warrants an Institutional Animal Ethics Committee (IAEC) with a member included from CPCSEA permits usage of laboratory bred animals like Guinea pig, Rabbit, Mice, Rats, Hamster etc.

The Medical Council of India in 2010 has replaced the usage of animal models with software for teaching and research purposes (60). Finally there is also a constant threat from organizations like People for Ethical Treatment of Animals (PETA), International Funding for Animal Welfare (IFAW) against animal experimentation.

4.20. NORMAL PHYSIOLOGICAL VALUES OF NEW ZEALAND WHITE RABBIT (NZWR)

Figure 5. New Zealand White Rabbit / *Oryctolagus cuniculus*

The following values may alter due to factors such as animal care, breeding, housing and environmental programs (61)

PARAMETER	VALUE
Body temperature	36-40 °C /100.4-104F
Heart Rate (beats per minute)	130-325
Respiration rate (per minute)	30-60
Weight range Adult male	2-6 kg
Weight range Adult female	2-6 kg
Weight Neonate	30-80 grams
Water Consumption (daily)	100-600 mL
Food Consumption (daily)	100-300 grams
Life Span	5-8 years
Sexual Maturity Age	4-6 months
Estrous cycle frequency	Induced
Duration of Estrus	NA
Gestation period	29-35 days
Breeding life	3 years

Table 3. Physiological values in New Zealand White Rabbit

4.21. RABBIT AS A SEPTIC SHOCK MODEL

- Xi Liu et al. developed a septic shock model using rabbits by injecting 0.6 mg per kg of lipopolysaccharide, *Escherichia Coli* serotype O111: B4 into the right jugular vein followed by Shenfu injection a Chinese herbal formulation (62).
- Chahim et al. demonstrated changes in Schwartzman phenomenon by instilling sterile culture of gram negative bacillus, *Salmonella typhosus* intra dermally followed by a consecutive provocative dose (63).
- Zi-Sen Zhang et al. developed an endotoxic septic shock in rabbit by administering LPS at 1mg per kg intravenously through the marginal ear vein, analyzed organ specific changes due to vascular reactivity by iNOS and endothelin-1 (64).
- Wu X et al. used caecal ligation and puncture method for inducing sepsis for studying the time of intervention using intra venous saline and Nor Epinephrine (NE) (65).
- Urosepis model resulting from Upper Urinary Tract Infection were made by ligating the ureter and injecting an uniform dosage of 9.0×10^8 cfu/mL standard Escherichia Coli into the renal pelvis at 2 mL per kg (66).
- Ma S et al. manipulated endo toxemia by intravenously injecting 60 µg/kg dissolved in 10 ml of saline to study hemodynamic parameters and multi organ damage (29).

- Jian-bo Yu et al. subjected the animals to intravenous injection pf 0.5 mL (5 mg/kg) LPS (L2630,sigma,USA) to create endotoxic shock induced Acute Kidney injury (67), (68).
- Chai-Zhi Sun et al. studied the effect of Dobutamine on lung aquaporin5 in an endotoxic shock model created by injecting intravenous LPS (600 μg/kg, Escherichia coli-serotype O_{55}:B_5, Sigma, USA) (69).
- Tao Yu et al. used a endotoxic shock model by intravenous injection of *Escherichia Coli* LPS (O55:B5; Sigma Chem Co, St. Louis, Mo) over a period of 30 s (70).
- Haiyang Wu et al. produced an uroseptic shock model with a combination of artificially increasing intra pelvic pressure and bacterial infection. They used mini lap approach to ligate the distal end of ureter followed by cannulating the proximal end and advancing the catheter into the renal pelvis. *Escherichia Coli* Strain at 2 ml per kg were delivered into the renal pelvis in the increasing concentration of 1.5×10^8, 3×10^8, 6×10^8, 9×10^8 cfu/ mL (71).
- Jia-Lin liang et al. studied the effect of IL-ß on vascular reactivity by injecting LPS (Sigma;O111B4) through marginal ear vein (72).

5. MATERIALS REQUIRED

1. White (Albino) New Zealand White Rabbit / *Oryctolagus cuniculus*
2. Anesthetic drugs
    a. For induction – Ketamine & Xylazine
    b. For maintenance – Ketamine & Midazolam
3. Interventional drugs
    a. Nor adrenaline
    b. Phentolamine
4. Freshly isolated *Escherichia coli* sample from the Department of Microbiology, Christian Medical College, Vellore
5. Smiths medical Graseby™ 2100 syringe pump
6. BD Insyte-W™ intravenous cannula 24GA
7. 50 mL disposable syringe
8. 1 mL disposable syringe
9. Micropipette cut ends
10. iPeX™ BKT-170 pressure Transducer
11. CMC data acquisition system (CMC DAQ)
12. Smiths medical C-Fusor® 500 reusable Pressure infusor
13. Respiratory belt
14. ECG hooks
15. Oxygen cylinder
16. Oxygen mask with connector
17. Clinical thermometer

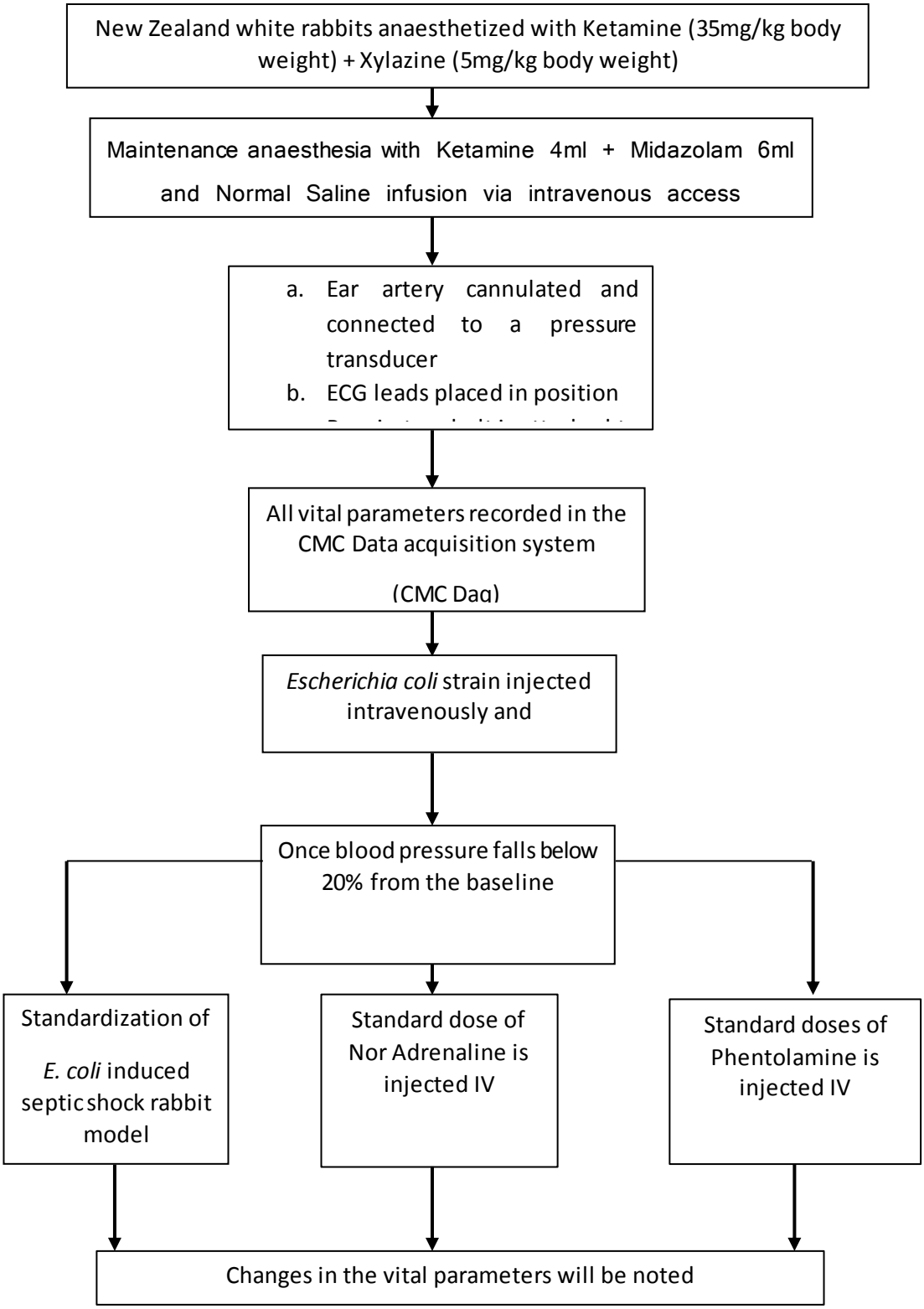
18. Abbott i-STAT 300 handheld Arterial Blood Gas (ABG) analyzer
19. Abbott i-STAT test cartridges EC8+
20. Abbott i-STAT printer
21. Sterile towel
22. Sterile gloves
23. Face mask
24. Surgical head cap
25. OT sterile surgical apron
26. Micropore
27. Cotton gauze
28. 20 mL test tube
29. Desktop No.7, keyboard, mouse, invertor
30. Sterile surgical instruments – Scissors, Scalpel blade 22
31. Inoculation (smear) wire loop
32. Alcohol burner

6. PARAMETERS RECORDED

1. Intra-arterial blood pressure
2. Electro cardiogram
3. Respiratory rate
4. Arterial blood gas analysis
5. Oral temperature

7. METHODOLOGY

7.1. DIAGRAMATIC ALGORITHM OF THE STUDY



7.2. ETHICS STATEMENT

New Zealand White Rabbits were purchased from Geniron biolabs, Bengaluru transported in an air-conditioned vehicle with food and water ad libitum. All the animals were housed in a pathogen free environment with 12 hour alternate dark/light cycle with free food and water ad libitum. All the animals were provided a proper standard care as formulated by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) under the CPCSEA guidelines for laboratory animal facility.

7.3. PREPARATION OF THE ANIMAL

New Zealand White Rabbits (NZWR) with coat color Albino were selected as the animal research model, which was obtained by Charles River Canada. Strict biosecurity practices were adhered when breeding the rabbits to ensure maintenance of best standards in genetics and health. All the animals were purchased from the College Animal House Facility, Christian Medical College, Bagayam, Vellore with Registration number: **88/PO/bc/1999/CPCSEA, 28.04.1999.** The gender of all the animal chosen for the experiment were females as they were most sensitive to the pathogens on the basis of our pilot study. Animals with body weight ranging from1.27 kilograms to 3.8 kilograms were randomly selected from the animal house on the previous day of the procedure. All the excess body fur of the animal were trimmed in order to prevent any conduction abnormalities while recording electrocardiogram. The dorsal aspect of both ears of the rabbit was shaved to avoid any difficulties in cannulation of artery and vein. Animal was weighed in an electronic weighing

apparatus on the previous day itself and put into overnight fasting. Overnight fasting of the animal aids in prevention of regurgitation and aspiration of gastric contents into the lungs thereby hindering aspiration pneumonitis. On the day of the procedure animal is weighed again to get the absolute body weight so that dosage of the drugs will be accurate matching the body weight.

All the animal studies were carried out in the sterile animal operating theatre facility provided by the College animal house, Christian Medical College, Vellore.

The drugs used for inducing anesthesia were Ketamine and Xylazine with the following dosage:

- Ketamine @ 35 mg per kg body weight
- Xylazine @ 5 mg per kg body weight

Each mL of Ketamine contains 50 mg, similarly each mL of Xylazine is 23.32 mg. Calculation of the induction dose was based the above mentioned values. The mode of injecting induction dose is intramuscular (im) in the gluteal region followed by adequate massaging to reduce pain at the injecting site. Usual time duration for the animals to get under the effect of anesthesia on an average is 2.8 minutes.

As soon as the animal gets under the influence of the anesthetic drug it was put in the left lateral position to keep the air way patent and to minimize aspiration.

Respiratory belt which were made in our department with a sensor attached to it was wrapped at the level of nipples to record the movements of the chest wall thereby calculating the rate of respiration. The respiratory belt was ensured to be dry as any wetness on it impairs proper recording. The other end of the belt was connected to CMC data acquisition system.

Hypodermic needle 21G with color code green were modified by bending its distal end at acute angles and were used as an ECG recording lead. Three ECG hooks, one each at the right, left infra axillary region and in the left groin fold were inserted. Each of the ECG hooks were connected to an ECG lead. The ECG lead clip were fixed to the hooks with no fur or muscle interfering the recording of electrical conduction of the heart. Red color coded lead were attached to the hook in the right infra axillary region, black color coded lead to the hook at the left infra axillary region. Gray color coded lead considered to be the earth were placed to the hook at the left groin fold. Placement of leads and hooks were same and followed for all the animal procedures to maintain uniformity. The other end of the ECG leads were connected to the CMC Data acquisition system. The output from CMC data acquisition system were connected to the department desktop number 7.

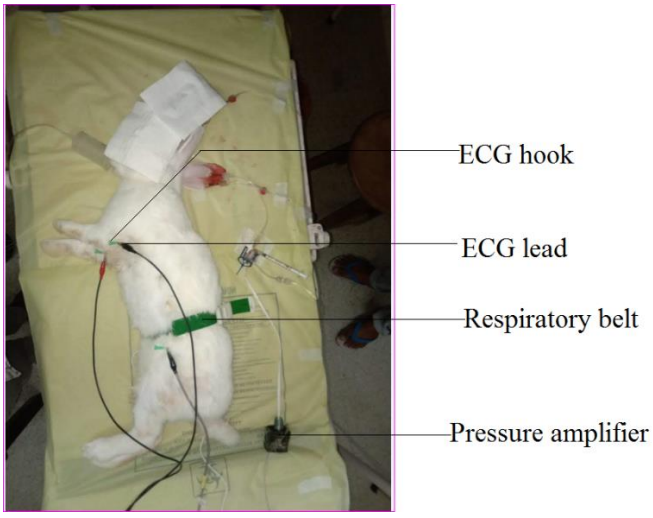


Figure 6. Anaesthetized animal connected with probe for recording all the vital parameters

7.4. MEASUREMENT OF ORAL TEMPERATURE

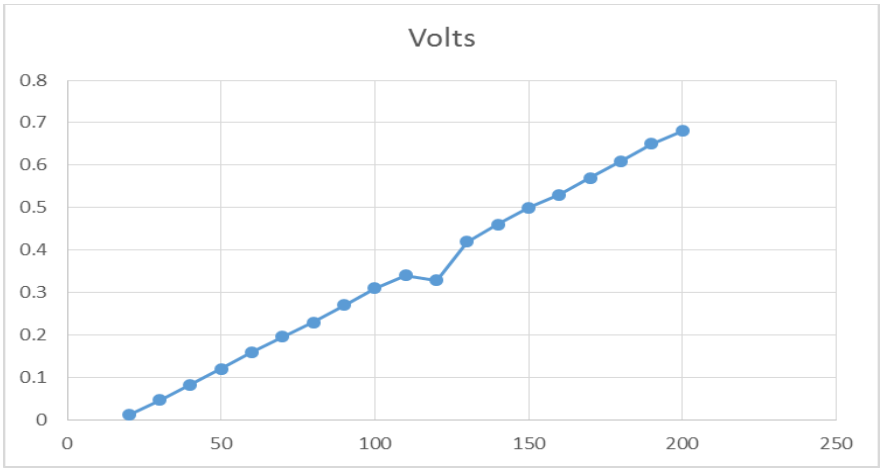
Digital thermometer MC-246 were used to measure the animal body temperature. The bulb of the thermometer were placed beneath the tongue for 2 minutes and the recording was noted. Immediately when the animal was under the effect of anesthesia the oral temperature was noted first and from there on half hourly recordings were made. Attempts were made to record rectal temperature as it is more reliable, but it was difficult since it disturbed the position of the animal and other recordings.

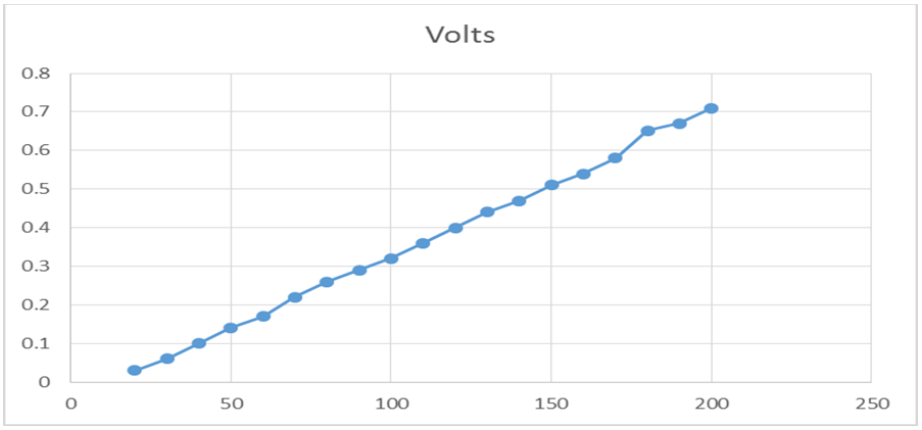
7.5. CALIBRATION OF PRESSURE TRANSDUCER AMPLIFIER KIT

iPeX™ non-pyrogenic invasive pressure monitoring kit model CMC-G, bkt-170 sterilized by Ethylene Oxide (EtO) and manufactured by BL Lifesciences Pvt. Ltd. were used for all the animal studies. The fluid used to fill the entire pressure invasive kit was normal saline with heparin added i.e., for every 10 mL normal saline 0.1 unit heparin was added. Presence of air bubbles will dampen the pressure recording. So the entire invasive pressure recording kit was fluid-filled to ensure strict absence of air bubbles throughout the recording system. Pressure recording end was connected to an empty 10 mL disposable syringe, another end was connected to the overhead hanging Smiths medical C-Fusor® 500 reusable Pressure infusor bag with the same fluid, and was maintained with a pressure of 300 mm Hg to prevent backflow.

Calibration of the pressure tranducer: The pressure transducer was linked to the CMC data acquisition system, pressure was raised gradually from 0 mm Hg to 200 mm Hg and corresponding amplitude were noted. Similarly pressure was decreased from 200

mm Hg to 20 mm Hg corresponding amplitude were noted. Calibration of the amplifier kit was done mandatorily for every five sets of experiments.





X axis - Pressure in mmHg

Y axis - Volts (mV)

Figure 7. Calibration of pressure transducer amplifier kit

PRESSURE(mm Hg)	VOLTS	PRESSURE (mm Hg)	VOLTS
20	0.012	200	0.71
30	0.046	190	0.67
40	0.082	180	0.652
50	0.12	170	0.58
60	0.16	160	0.54
70	0.196	150	0.51
80	0.23	140	0.47
90	0.27	130	0.44
100	0.31	120	0.4
110	0.34	110	0.36
120	0.328	100	0.32
130	0.42	90	0.29
140	0.46	80	0.26
150	0.5	70	0.22
160	0.53	60	0.17
170	0.57	50	0.141
180	0.61	40	0.1
190	0.65	30	0.06
200	0.68	20	0.03

Table 4. Calibration values for pressure transducer kit

7.6. CANNULATION OF THE BLOOD VESSELS

The animals were maintained in the left lateral position which not only prevented aspiration of the stomach contents into the air way, but also the right ear was plastered on the flat surface which enabled easier cannulation of the artery and vein as well as securing the BD Insyte-W™ intravenous cannula 24GA were the most convenient. Prior to cannulation all the intravenous cannula were flushed with heparin lock flush solution derived from porcine intestinal mucosa to maintain fluidity of the blood within the system and also to avoid any air bubbles.

A bright fluorescent lamp was placed beneath the ventral surface of the ear to enhance illumination and visibility of the blood vessels. Even reduction in the room temperature below 30°C caused constriction of the vasculature which made the cannulation extremely difficult. Hence the room temperature were strictly adhered to above 30°C. 70% ethanol were sprayed over the dorsal surface of the ear to sterilize but it also produced a slight vasodilation which made the visibility of the blood vessels clearer.

Cannulation were always started with the dependent aural vein due to practical difficulties. After insertion of the cannula into the vein sluggish flow of the blood were seen till the edge of the cannula, if not heparin solution diluted with normal saline were added to fulfill the void. The cannula were then connected to the Smiths medical Graseby™ 2100 syringe pump which contains Ketamine and midazolam for maintenance dose of anesthesia.

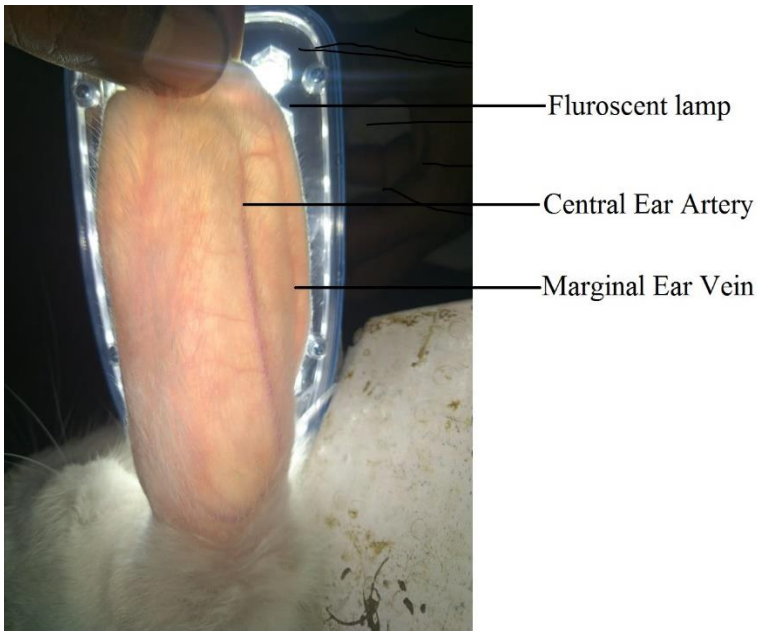


Figure 8. Illumination lamp used for cannulation of ear blood vessels

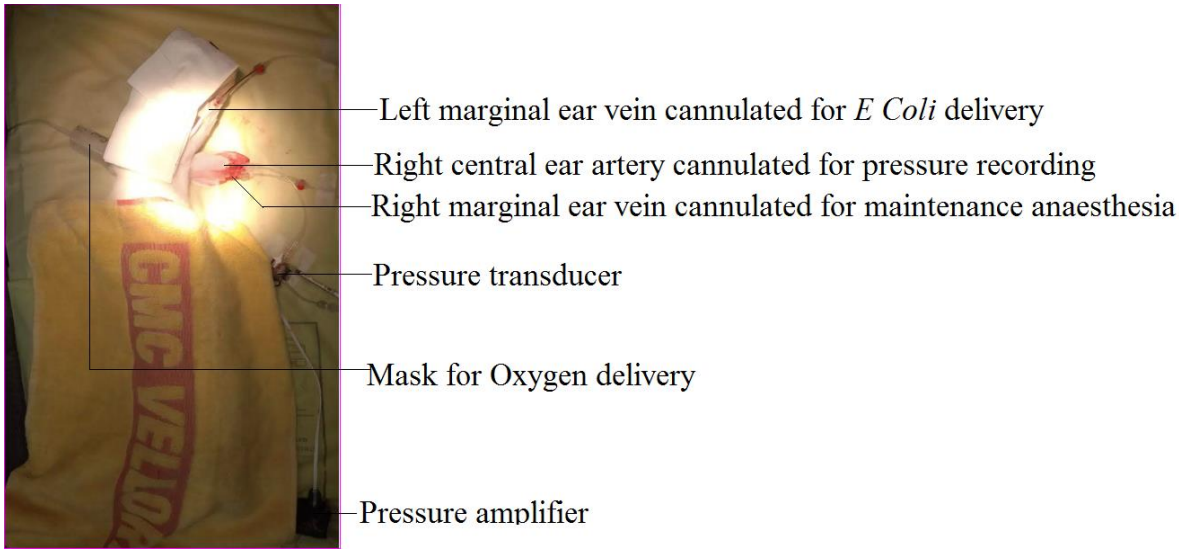


Figure 9. Cannulation of the central ear artery and marginal ear veins

7.7. PREPARATION OF MAINTENANCE ANESTHETIC DRUG AND DRUG DELIVERY SYSTEM

4 mL of ketamine along with 6 mL of midazolam made the maintenance anesthesia which were mixed adequately in a 50 mL disposable syringe attached to Smiths medical Graseby™ 2100 syringe pump. The connector from the syringe to the cannula were completely free from air bubbles, the end of the connector was fixed to a micro pipette end. The use of micro pipette cut end between the cannula and the connector was to enhance stable fixation to prevent any leakage of the drug neither allowing air to enter the system. The maintenance dose were constant for all the animal studies and dispersed at a steady rate of 4 mL per hour.

7.8. CANNULATION OF THE DEPENDENT ARTERY FOR BLOOD PRESSURE MEASUREMENT

After completing the cannulation of the right ear vein and connecting it to the maintenance anesthesia drug delivery pump ensured that the animal is under the complete influence of the drug in order to complete other procedures with ease. The dorsal surface of the right ear were again wiped with 70% ethanol to induce slight vasodilatation with the bright fluorescence lamp placed beneath to enhance the visibility of the central ear artery. 24 GA BD-Insyte-W™ cannula were inserted along the course of the artery, positioned of which was confirmed by sudden gush of bright red oxygenated arterial blood.0.2 mL to 0.3 mL of the arterial blood sample were collected in a disposable syringe flushed with heparin. After collecting the arterial blood sample the cannula end were fixed iPeX™ BKT-170 pressure transducer with a

micropipette cut end in between to ensure stability of the recording line. The lateral end of the pressure transducer was connected with overhead hanging Smiths medical C-Fusor® 500 reusable Pressure infusor bag with the same fluid were maintained with a pressure of 300 mm Hg to prevent backflow. The other end of the transducer were connected to the CMC Data acquisition system, corrections to match the atmospheric pressure were done by turning the three way knob only connected to the atmosphere. Again the previous set up were done, once the proper blood pressure wave form are seen in the monitor the plunger system of the transducer were pulled to establish flush test after which the recording of the blood pressure, electrocardiogram and respiratory rate were started. Oxygen were delivered nasally at a rate of 4-6 L per min with a custom made mask and connector. Eyes of the animal were covered with a sterile ocular pad made of surgical gauze.

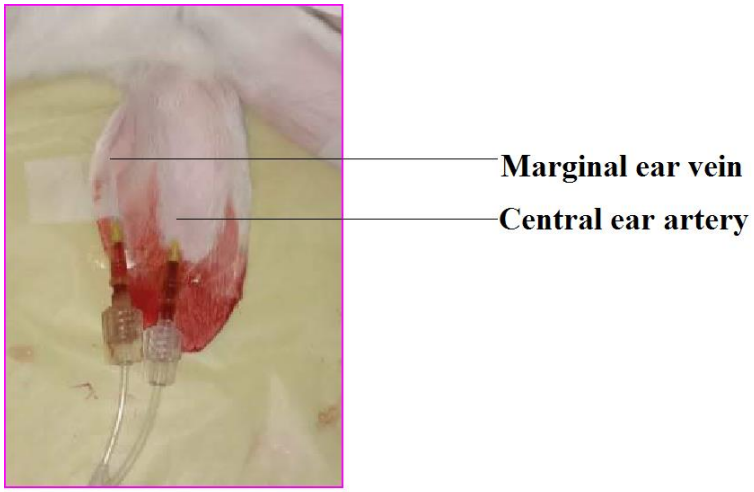


Figure 10. Central ear artery cannulated for measuring blood pressure

7.9. CANNULATION OF THE NON DEPENDENT MARGINAL EAR VEIN FOR FLUID ADMINISTRATION

The nondependent ear of the animal were used for cannulation of the veins for fluid delivery and maintenance anesthesia. One marginal ear vein were kept ideally for only for 4 mL of ketamine along with 6 mL of midazolam (maintenance dose anesthesia) which were mixed adequately in a 50 mL disposable syringe attached to Smiths medical Graseby™ 2100 syringe pump. The maintenance dose were delivered constantly at a steady rate of 4 mL per hour for all the animals. The other marginal vein were cannulated and connected to another Smiths medical Graseby™ 2100 syringe pump that delivered Normal saline as a fluid resuscitation at a dosage of 4 mL per kg per hour.

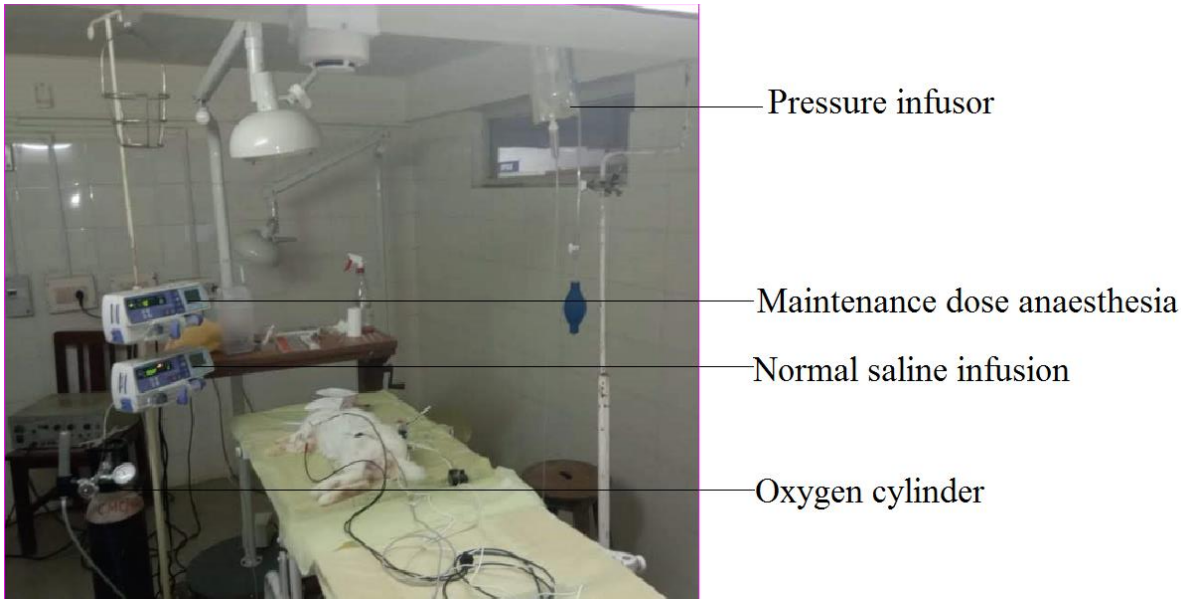


Figure 11. Whole experimental set up

7.10. ARTERIAL BLOOD GAS ANALYSIS PRIOR TO *ESCHERICHIA COLI* ADMINISTRATION

PARAMETERS CHECKED

1. Serum Sodium (Na^+) in mmol/L
2. Serum Potassium (K^+) in mmol/L
3. Serum Chloride (Cl^-) in mmol/L
4. Total Carbon Di Oxide (CO_2) in mmol/L
5. Blood Urea Nitrogen (BUN) in mg/dL
6. Blood Glucose (Glu) in mg/dL
7. Hematocrit (Hct) % PCV
8. pH
9. Partial pressure of CO_2 (PCO_2) in mmHg
10. Bicarbonate (HCO_3) in mmHg
11. Anion Gap (AnGap) in mmol/L
12. Hemoglobin (Hb) in g/dL * Via Hct

Prior to the procedure the Standard i-STAT cartridge were inserted into the analyzer and quality check of the apparatus were performed for every usage. The bar code on the test cartridges were scanned by the scanner attached to the analyzer, unrecognized cartridges were not used.

Once proper recording of all the parameters were established the blood sample collected for ABG analysis were filled in the well of the Abbott i-STAT test cartridges

EC8+ cartridge. The door of the cartridge was closed after adequate filling of the well followed by insertion into the Abbott i-STAT 300 handheld Arterial Blood Gas (ABG) analyzer. It usually takes 2 to 3 minutes for the blood sample to be analyzed and the result of which were displayed in the monitor. The monitor of the analyzer was the connected to an Abbott i-STAT printer and the results were printed and preserved.

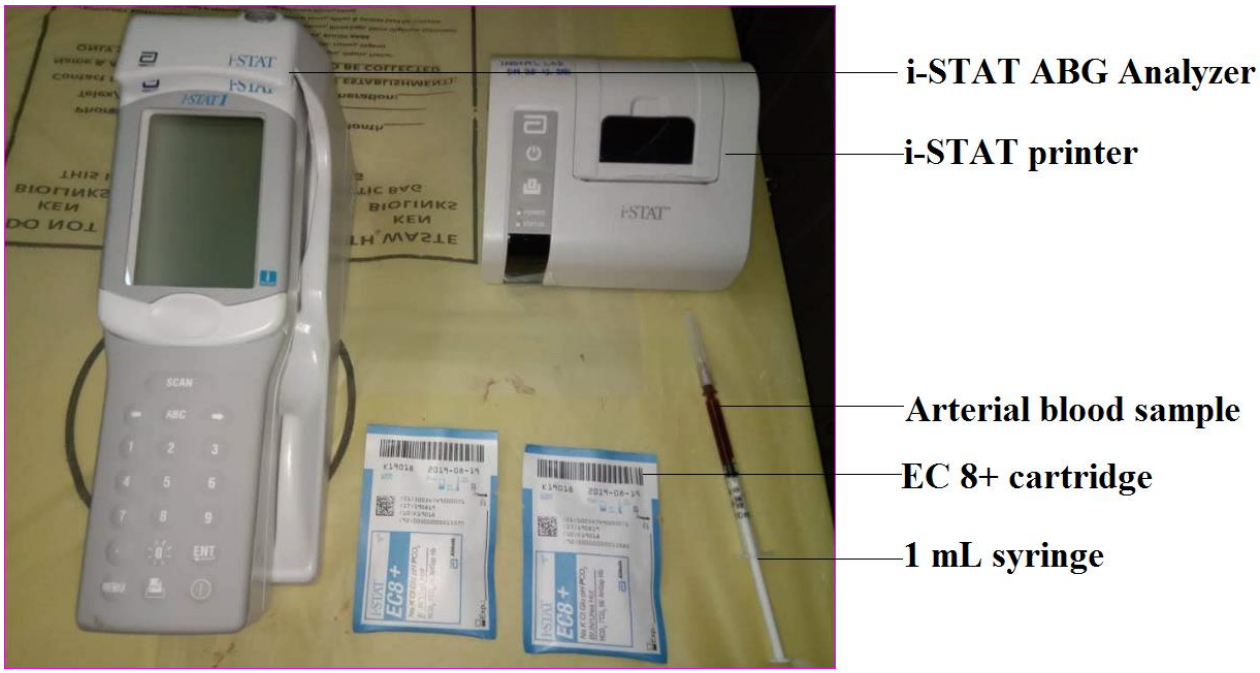


Figure 12. I-STAT ABG analyzer kit

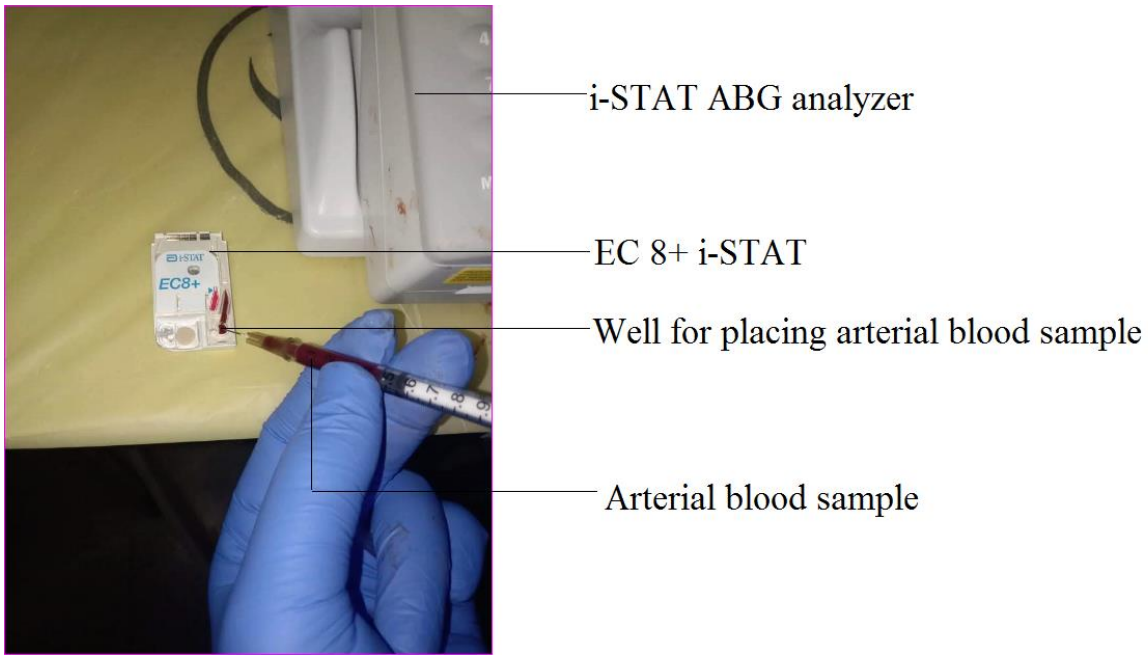


Figure 13. Charging the EC 8+ i-STAT cartridge for ABG analysis

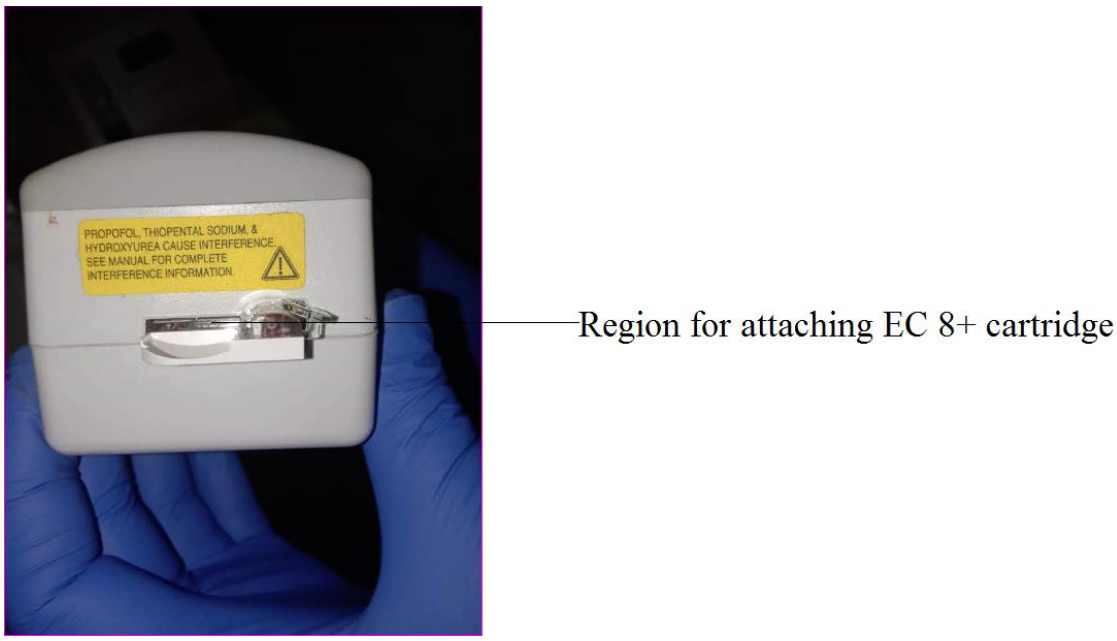


Figure 14. Attachment of EC 8+ i-STAT cartridge

7.11. *ESCHERICHIA COLI* PREPARATION

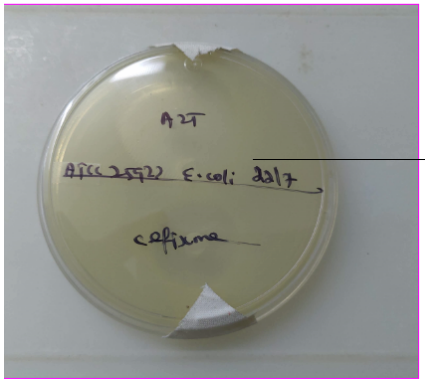
Fresh pure culture of *Escherichia Coli* were obtained from the Department of Microbiology, Christian Medical College, Vellore in a sterilized petri dish. The pure culture plate were maintained in a special culture hood specially designed for preparing the inoculum.

McFarland standard were utilized to approximately quantify the number of bacteria's present in the suspension prepared. McFarland standard were prepared by mixing 1% barium chloride ($BaCl_2$) and 1% sulfuric acid (H_2SO_4) which forms barium sulfate precipitates thus producing turbidity. The turbidity of the test suspension prepared were compared with that of the McFarland standard. The McFarland Standard were prepared in a test tube similar to the one that is used to prepare the test solution. The tube containing the standard were shaken well before the procedure to secure uniform distribution of the precipitates and were sealed to prevent evaporation.

McFarland Standard	**1% H_2SO_4 in mL**	**1% $BaCl_2$ in mL**	**Approximate bacterial suspension per mL**
0.5	9.95	0.05	1.5×10^8
1.0	9.9	0.1	3.0×10^8
2.0	9.8	0.2	6.0×10^8
3.0	9.7	0.3	9.0×10^8
4.0	9.6	0.4	1.2×10^9
5.0	**9.5**	**0.5**	**1.5×10^9**
6.0	9.4	0.6	1.8×10^9
7.0	9.3	0.7	2.1×10^9
8.0	9.2	0.8	2.4×10^9
9.0	9.1	0.9	2.7×10^9
10.0	9.0	1	3.0×10^9

Table 5. McFarland standard values

Normally 2.5 McFarland units were used for culture media performance testing and antimicrobial susceptibility testing. For inducing septic shock pilot studies were done using increasing doses of McFarland units from 0.5 to 5. With the results from the pilot study 5 McFarland units were used to induce sepsis in the animal which resulted in decreasing the blood pressure by 20% from the baseline. An aluminum alcohol burner was used for flame sterilization of the inoculation wire loop made up of Ni-chrome. The smear loop after heated in the flame were used to scoop the *Escherichia Coli* from the fresh pure culture plate. Two to three scoops of the organism were mixed with normal saline for the preparation of the inoculum and used for each animal studies. Nutrient broth were tried as a medium of suspension but deployed since it helped in the growth and proliferation of the organisms thus giving false number of bacteria's. Under good lightning the test inoculum were compared with that of the McFarland standard, if the test solution is too light some more scoops of organisms were added. If the test solution is too dark compared to the standard, dilution was done using normal saline with a sterile pipette.



Freshly isolated *Esherichia Coli* sample in a petri dish

Figure 15. Petri dish with *Escherichia Coli*

7.12. PRECAUTIONS PURSUED

- McFarland standard tubes were tightly sealed and kept in the dark environment since they are sensitive to light and air.
- The level of the solution in the McFarland standard tubes were checked periodically, any loss of volume due to evaporation were discarded and a new standard was prepared.
- If the turbidity were not uniform or any precipitates if noted the standard was discarded.

7.13. QUALITY CONTROL

Accuracy of the McFarland Standards were checked using spectrometer with 1 cm light path by the Department of Microbiology.

7.14. STORAGE

McFarland Standards were placed in upright position at 4°C to 25°C.

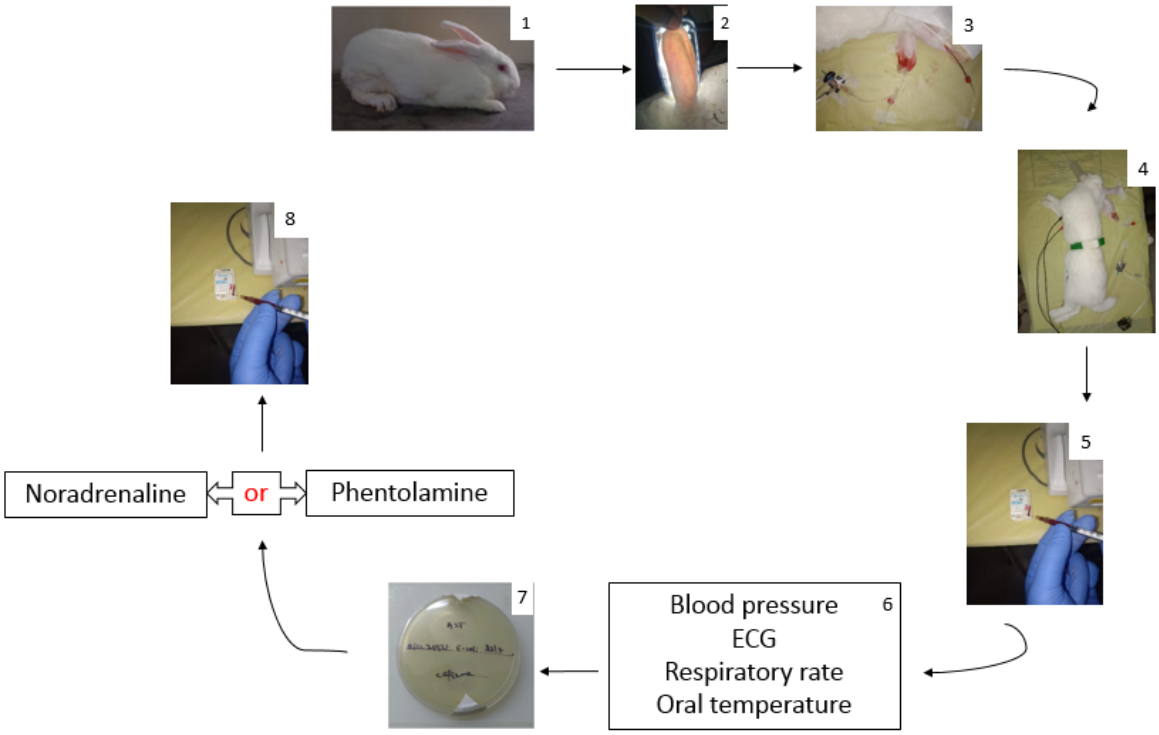


Figure 16. Entire Methodology

1. New Zealand White Rabbit
2. Enhancing the visibility of aural blood vessels
3. Cannulation of the blood vessels
4. Anaesthetizing the animal and establishing the connections for recording the vitals
5. ABG analysis
6. Vitals recorded
7. Escherichia Coli administration
8. Post sepsis ABG analysis

7.15. *ESCHERICHIA COLI* ADMINISRATION

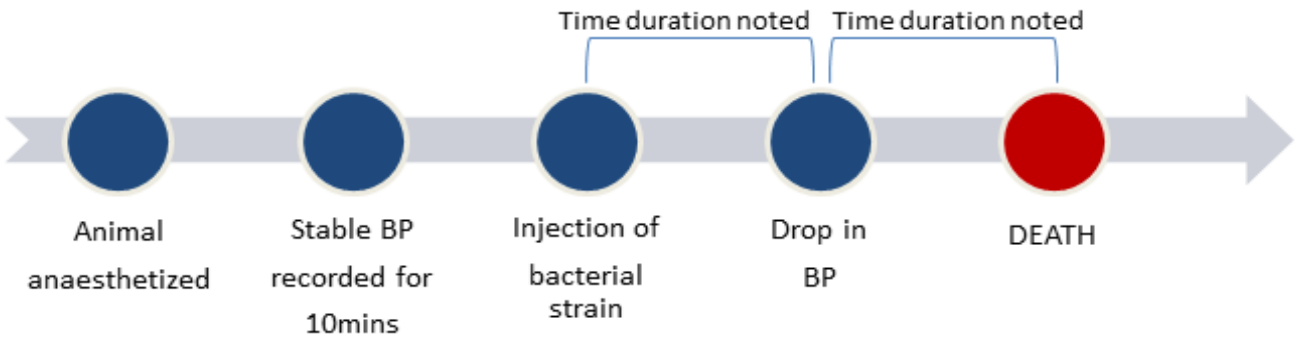


Figure 17. Time line for E Coli administration

When the Rabbit is completely under the influence of the anesthesia all the vital parameters Blood pressure, respiratory rate, ECG were recorded for a period of 10 minutes to get the baseline values. As soon the blood pressure falls below the 20% of the baseline freshly prepared *Escherichia Coli* @ 2 mL per kg were given as an intravenous bolus through any of the nondependent ear marginal vein.

7.16. NOR ADRENALINE INTERVENTION

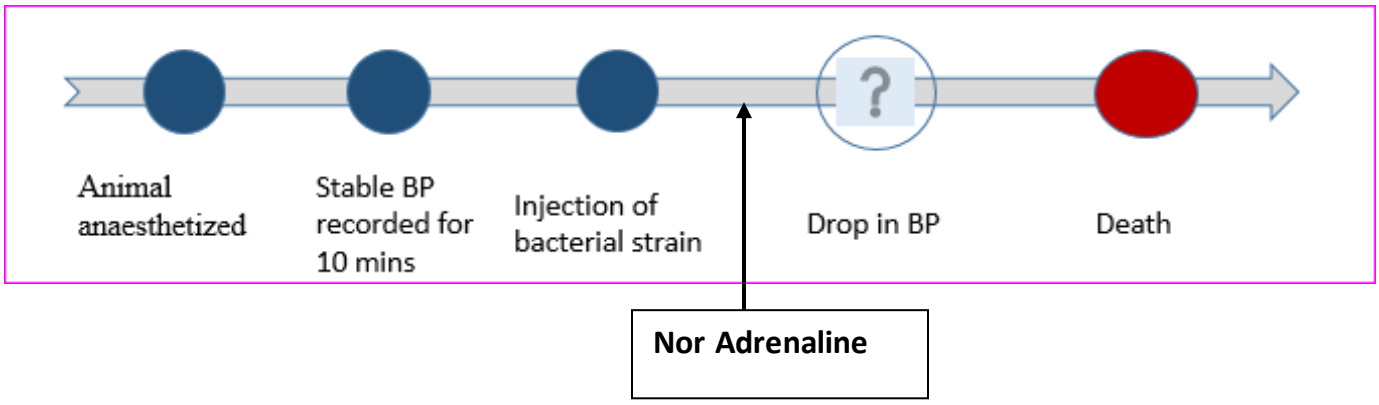


Figure 18. Time line for Noradrenaline administration

In a set of animals (n=6) after administration of the *Escherichia Coli* the vitals were recorded for a period of 20 minutes and alpha agonist Nor Adrenaline were given intravenously at a dose of 0.1 µ/kg/min and time to death were noted.

7.17. PHENTOLAMINE INTERVENTION

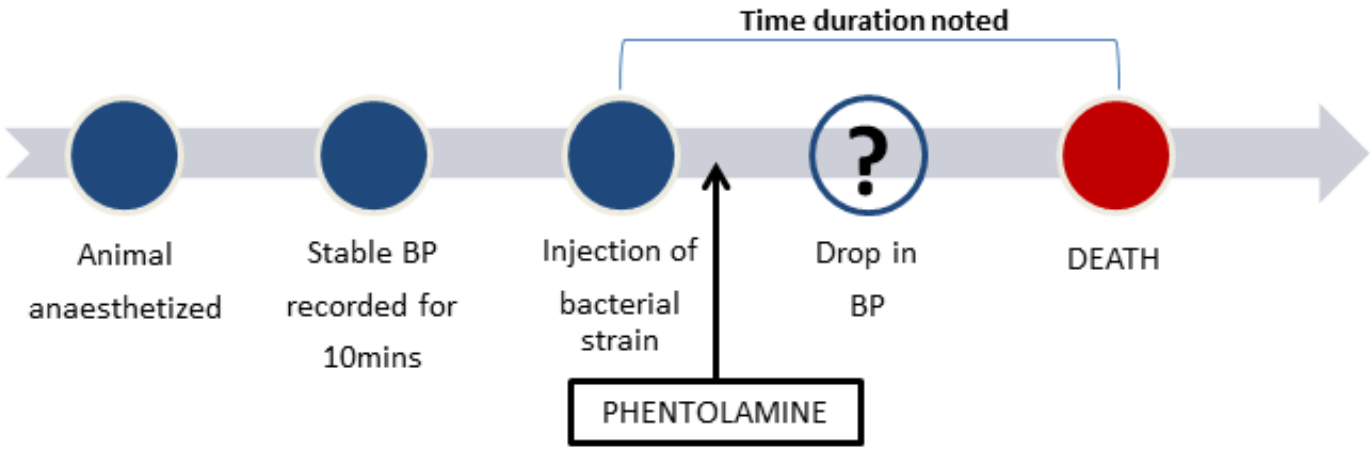


Figure 19. Time line for Phentolamine administration

In another set of animals (n=4) after administration of the *Escherichia Coli* the vitals were recorded for a period of 20 minutes and alpha antagonist Phentolamine were given intravenously at a dose of 0.1 mg/kg/hour and time to death were noted.

7.18. ABG ANALYSIS POST SEPSIS

Just prior to the death of the animal another arterial blood sample were collected as per the method described previously and post sepsis ABG reading were noted for all the animals.

7.19. PRESERVATION OF BLOOD SAMPLES

After death of the animals 10 mL of blood were taken from the ventricles of the heart approached on the left side of the chest through the diaphragm using 21G needle. The collected blood sample were then centrifuged at 2400 rpm for 10 minutes and the supernatant were stored at -20°C using Eppendorf g-safe® 1.5 mL tubes. The supernatant was stored for future analysis of serum biochemical values.

7.20. PRESERVATION OF VITAL ORGAN SPECIMENS

Immediately after the death of the animal all the cannulations were removed from the vessels. The vital organs were removed and preserved in separate containers with 10% buffered formalin for future analysis and histopathological examination.

7.21. DISPOSAL OF THE ANIMAL CARCASSES

The animal carcasses after all the procedure were disposed through our animal disposal team. Ken bio links private limited used incineration as the method of disposing the carcasses.

8. STATISTICAL ANALYSIS

- All the statistical analysis were done using SPSS and PSPP software.
- The values are expressed as Mean ± SD.
- Kruskal- Wallis H (KWH) test was used to analyzing the test of significance between the three study groups.
- Wilcoxon Signed Ranks (WSR) test were utilized to study the test of significance before and after intervention within the same group.
- A p-value of <0.05 were taken as significant.

9. RESULTS

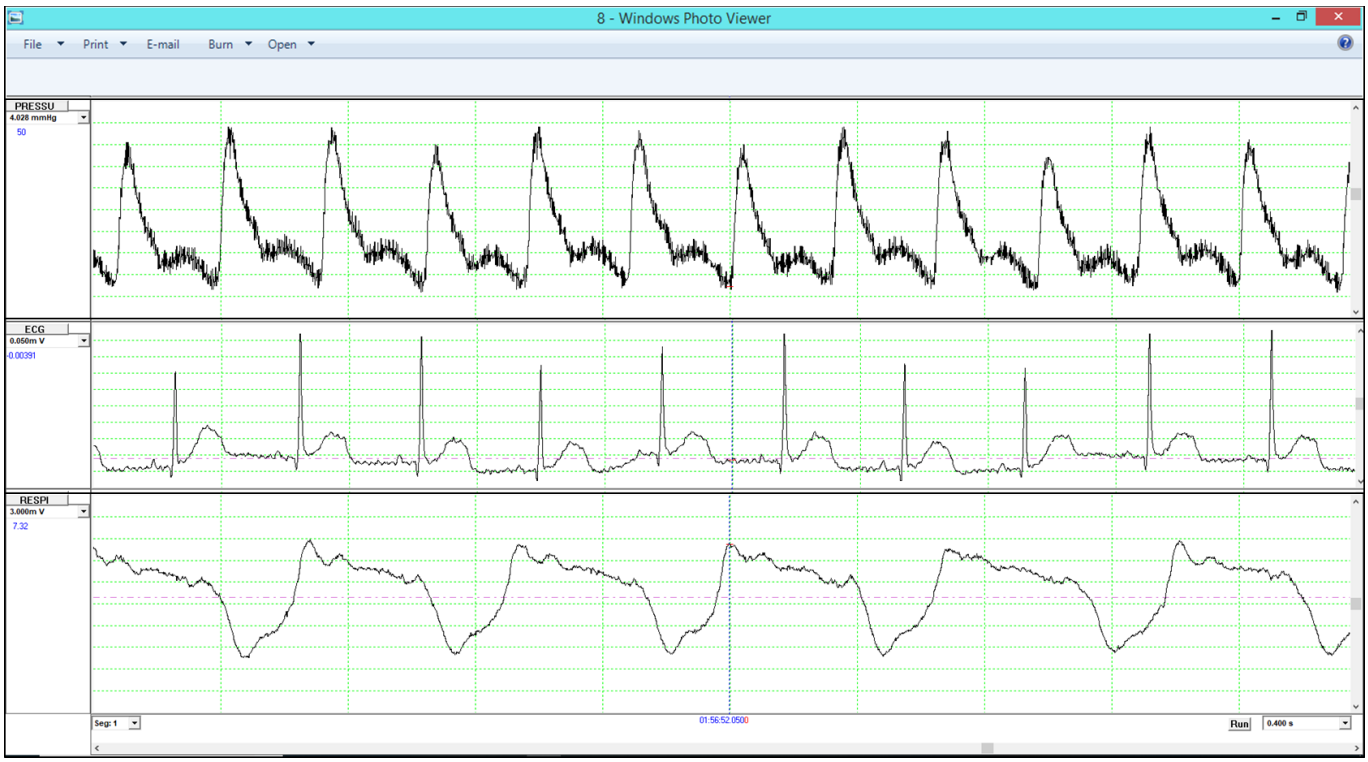


Figure 20. Raw data of all the parameters recorded

Channel 1-Blood pressure recording

Channel 2- ECG recording

Channel 3- Respiratory rate recording

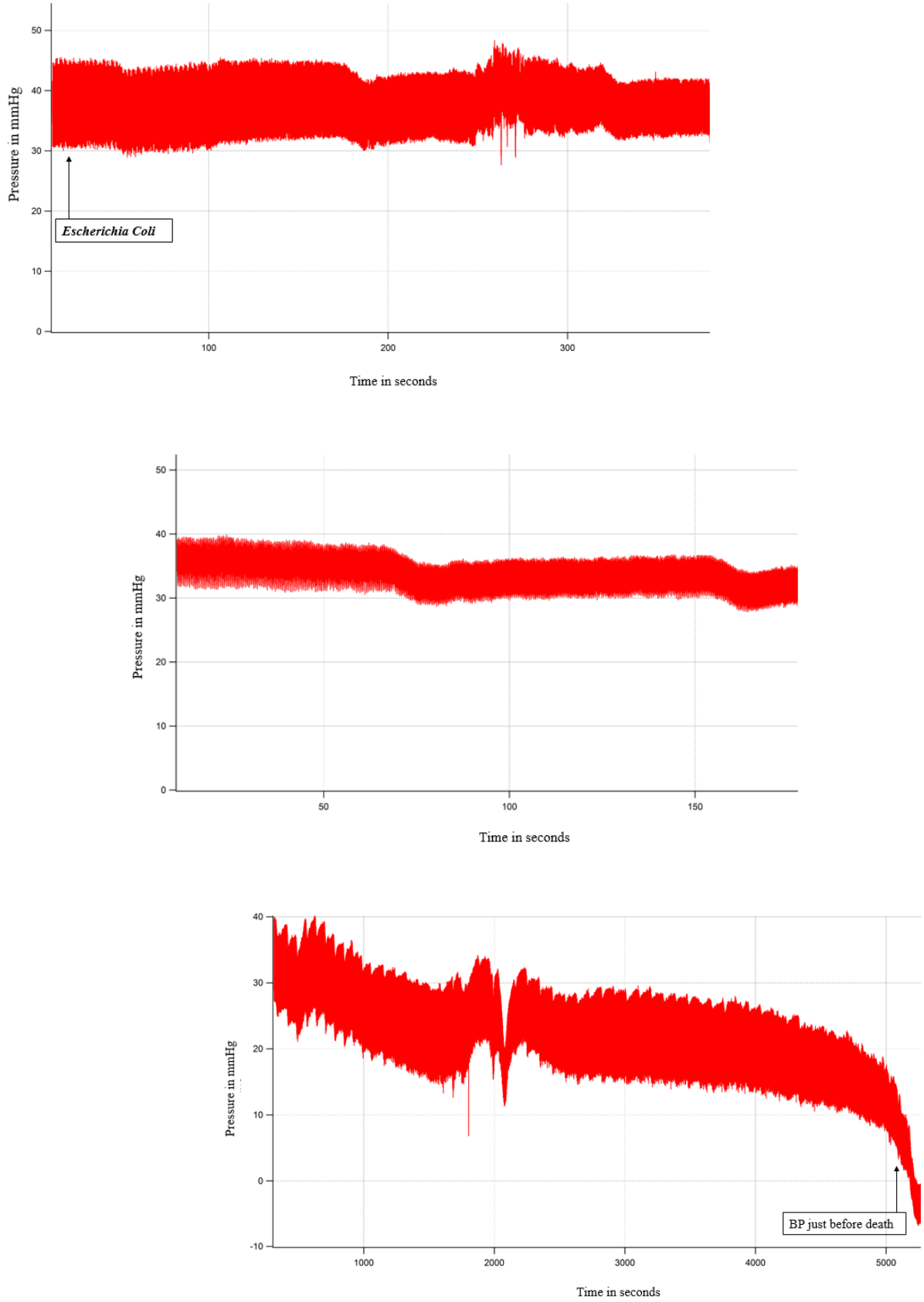


Figure 21. Blood pressure recording after administration of *Escherichia Coli*

***Escherichia Coli* + Noradrenaline**

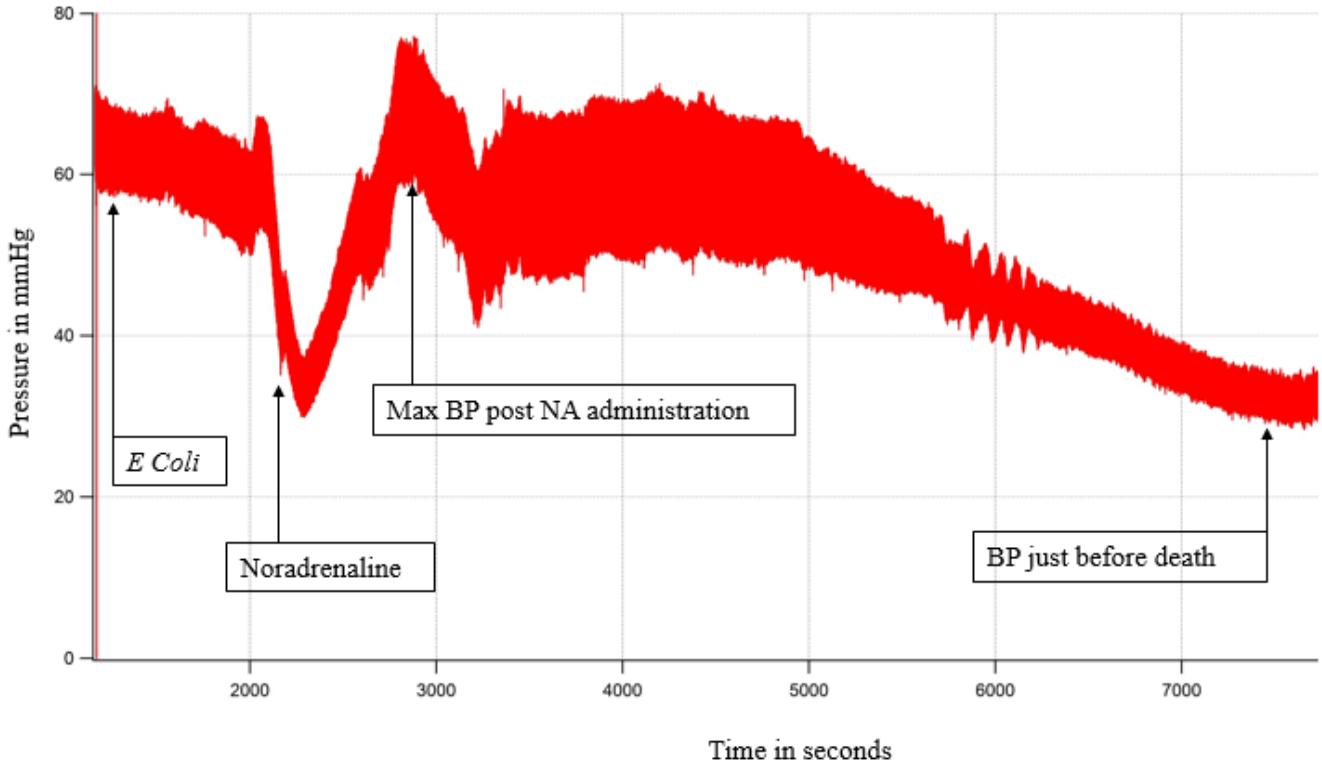


Figure 22. Blood pressure recording after intervention with an alpha agonist

***Escherichia Coli* + Phentolamine**

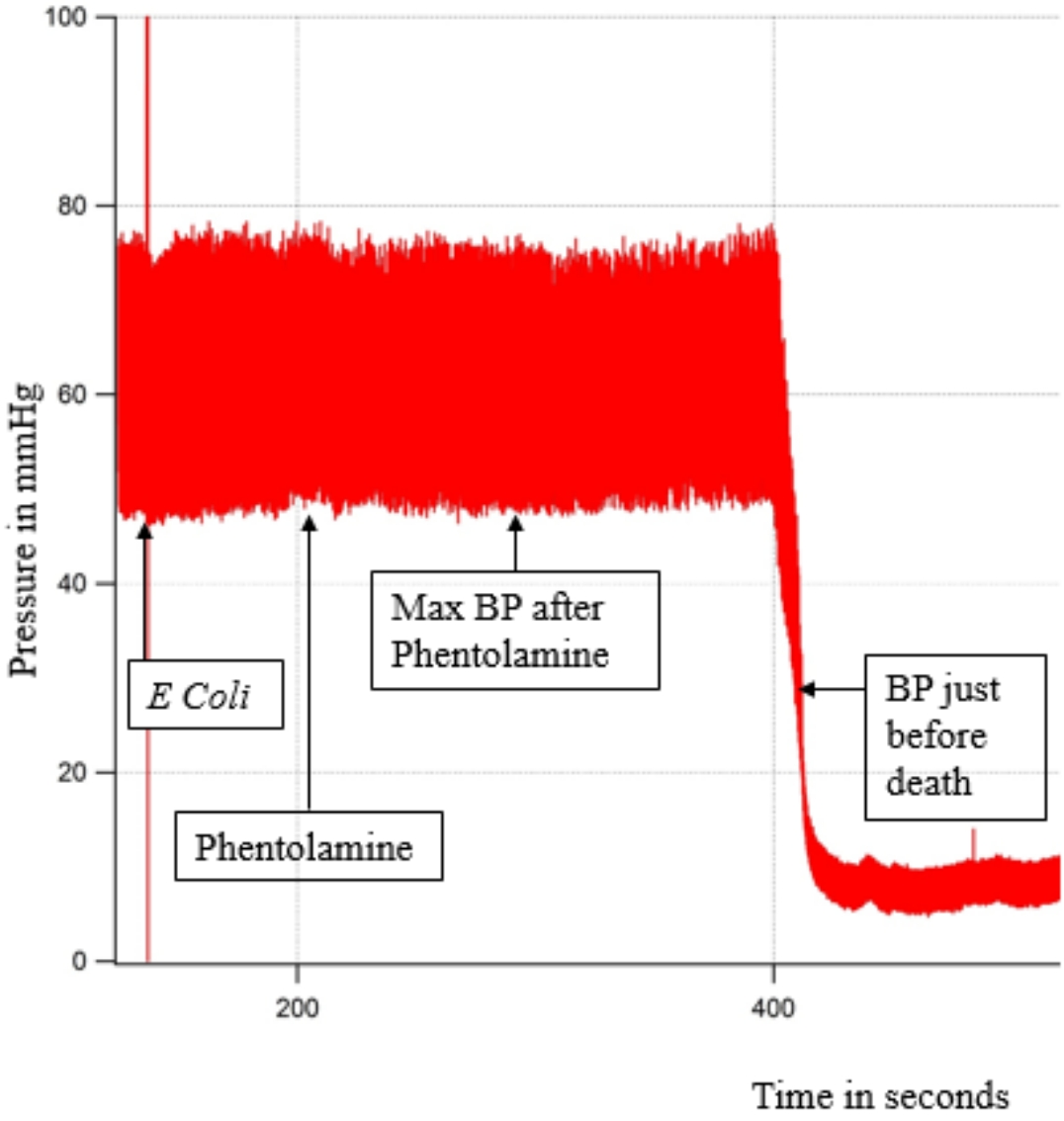


Figure 23. Blood pressure recording after intervention with an alpha antagonist

MEAN ARTERIAL PRESSURE

Group of animals which received *Escherichia Coli* alone

MAP dropped significantly from 37.13 ± 15.06 to 26.85 ± 15.06 with a p value of **0.015,** n=10, with WSR test and is significant statistically.

Group of animals which received *Escherichia Coli* and Nor Adrenaline

MAP dropped from 53.95 ± 11.35 to 41.57 ± 10.59 after administration of *Escherichia Coli*. Intervention with Nor Adrenaline increased MAP from 41.57 ± 10.59 to a maximum of 66.77 ± 25.05. MAP just before death of the animal was 35.60 ± 17.04. MAP before and after intervention with Nor Adrenaline was analyzed with WSR test which gave a p value of **0.028**, n=6, and is statistically significant.

Group of animals which received *Escherichia Coli* and Phentolamine

MAP dropped from 61.22 ± 8.94 to 60.54 ± 4.66 after administration of *Escherichia Coli*. Additive effect of *Escherichia Coli* with Phentolamine reduced the MAP to 37.13 ± 10.89. MAP before and after intervention with Phentolamine was analyzed with WSR test which gave a p value of 0.715, n=4, and is not significant statistically.

Percentage change in MAP between the above three groups were analyzed with KWH test which gave a p value of **0.039** and is statistically significant.

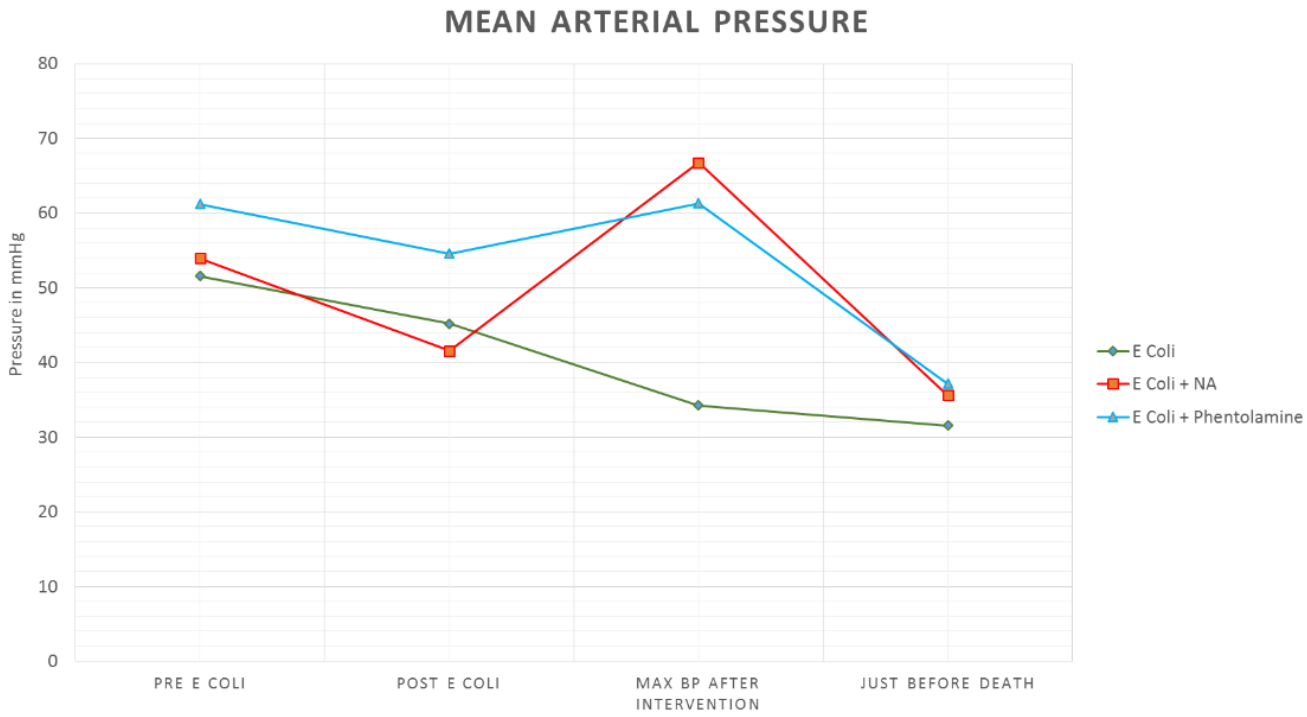


Figure 24. Comparison of MAP between all the groups of animals

PULSE PRESSURE (PP)

Group of animals which received Escherichia Coli alone

PP dropped from 15.15 ± 6.74 to 12.38 ± 11.55, analysis with WSR test gave a p value of 0.110, n=10, which is not significant statistically.

Group of animals which received *Escherichia Coli* + Nor Adrenaline

PP dropped from 21.90 ± 8.13 to 20.26 ± 7.43 after administration of *Escherichia Coli*. Intervention with Nor Adrenaline reduced the PP from 20.26 ± 7.43 to 16.08 ± 7.77. PP just before the death of the animal was 8.58 ± 5.57. PP before and after intervention with Nor adrenaline was analyzed with WSR test which gave a p value of 0.463, n=6 and is not significant statistically.

Group of animals which received *Escherichia Coli* + Phentolamine

PP dropped from 24.33 ± 6.34 to 22.94 ± 9.24 after administration of *Escherichia Coli*. Additive effect of *Escherichia Coli* with *Phentolamine* reduced the PP to 17.80 ± 0.80. PP before and after intervention with Phentolamine was analyzed with WSR test which gave a p value of 0.465, n=4 and is significant statistically.

Percentage change in PP in the above three groups were analyzed with KWH test which gave a p value of 0.707 and is not significant statistically.

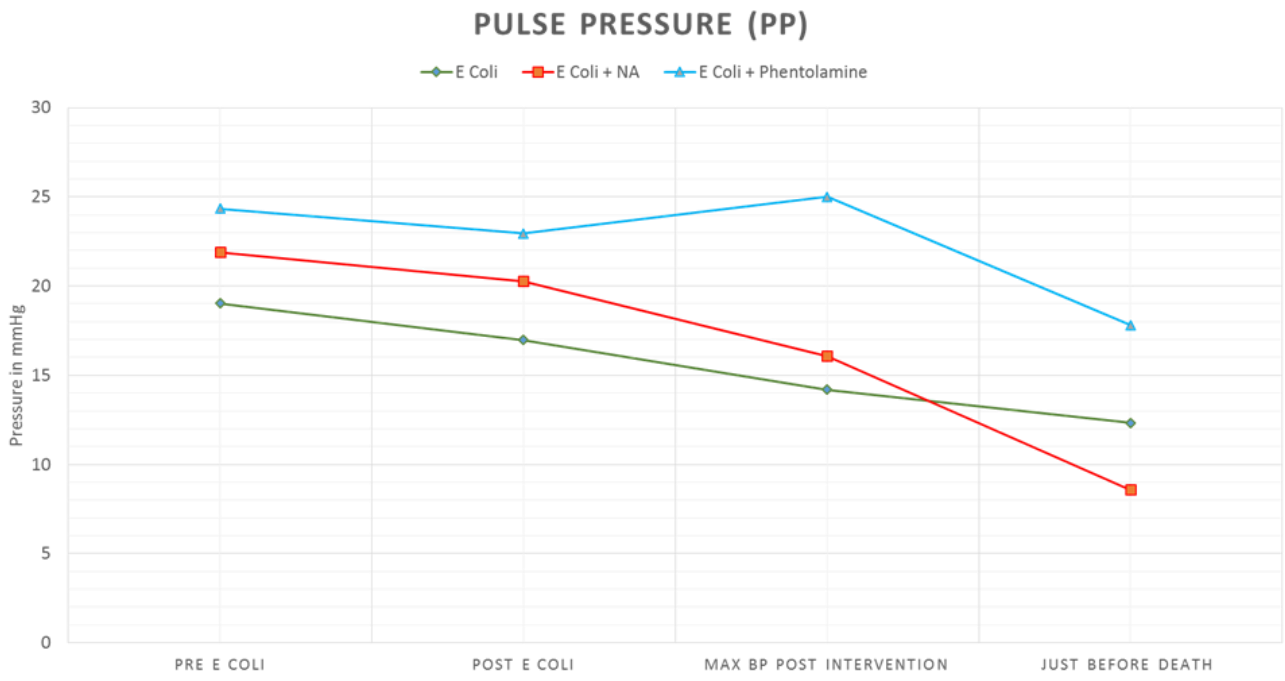


Figure 25. Comparison of PP between all the groups of the animals

TIME TO DEATH

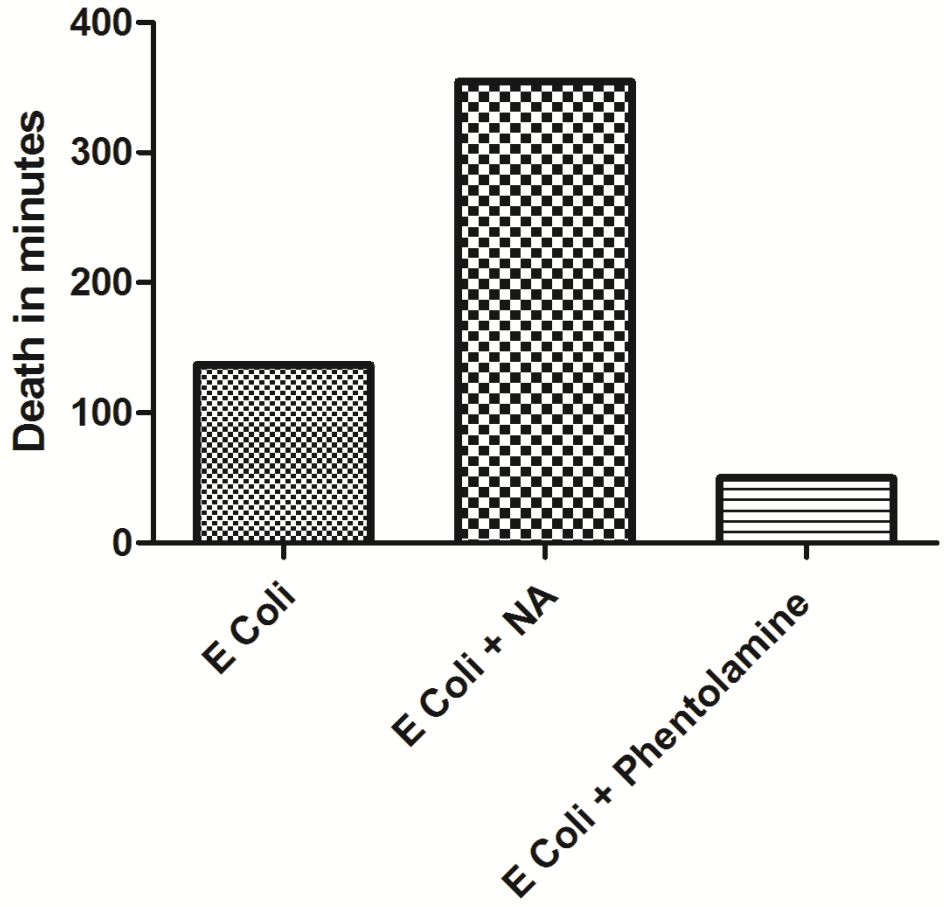


Figure 26. Bar diagram comparing the time to death in all the groups of the animals

Animals that received *Escherichia Coli* alone for developing Septic shock model died in 136.80 ± 22.190 minutes of administration. Animal group which received E Coli + Nor Adrenaline died in 354 ± 58.674 minutes following intervention. Animal group which received E Coli + Phentolamine died in 49.75 ± 26.862 minutes following

intervention. Non parametric analysis between these groups done with KWH test showed p value of **0.000** which is statistically significant.

BODY TEMPERATURE

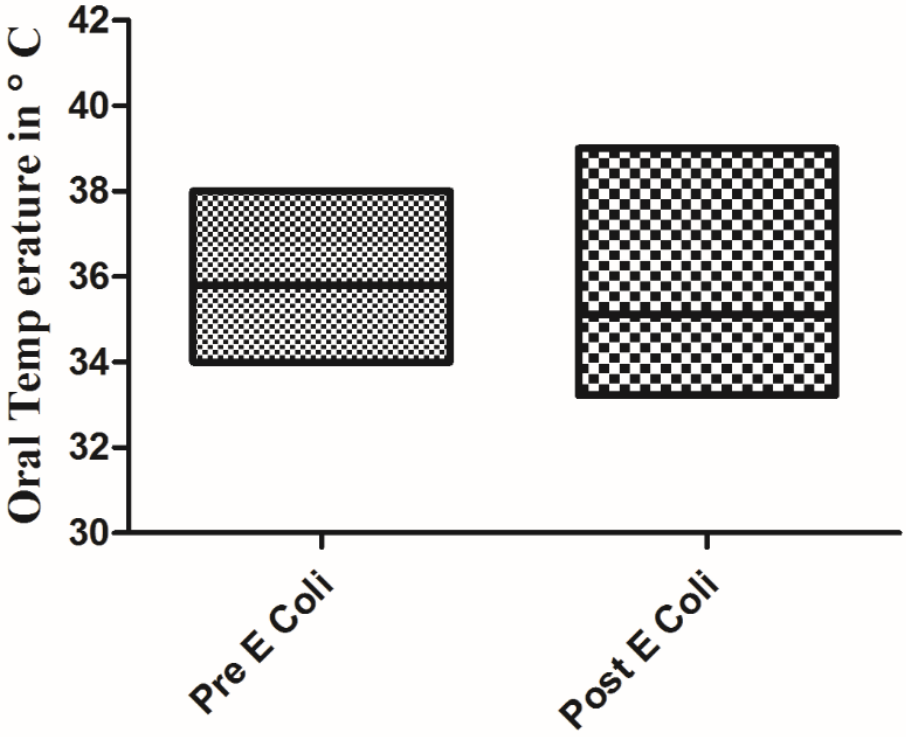


Figure 27. Oral temperature before and after sepsis

The body temperature of the animals decreased from 35.80 ± 1.32 to 35.12 ± 1.48 after the development of sepsis with Escherichia Coli with a p-value of **0.06** which is not significant statistically with KWH test.

ABG ANALYSIS

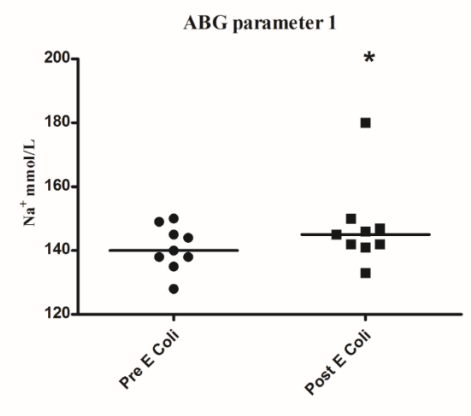


Figure 28. Serum sodium before and after sepsis

Serum sodium increased from 140.30 ± 6.783 to 146.50 ± 12.978 after administration of *Escherichia Coli* with a p-value of **0.040** which is significant statistically with WSR test (n=10) .

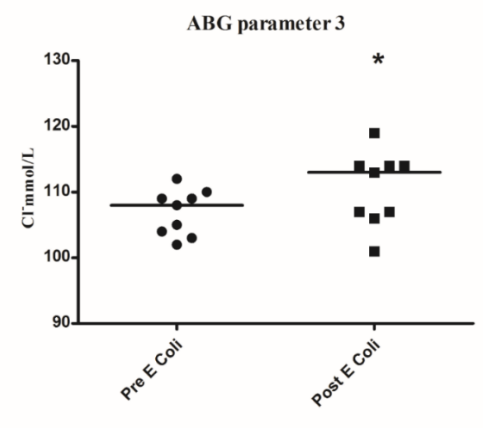


Figure 29. Serum chloride before and after sepsis

Serum Chloride increased from 106.70 ± 3.335 to 110.40 ± 5.296 after administration of *Escherichia Coli* with a p-value of **0.012** which is significant statistically with WSR test (n=10).

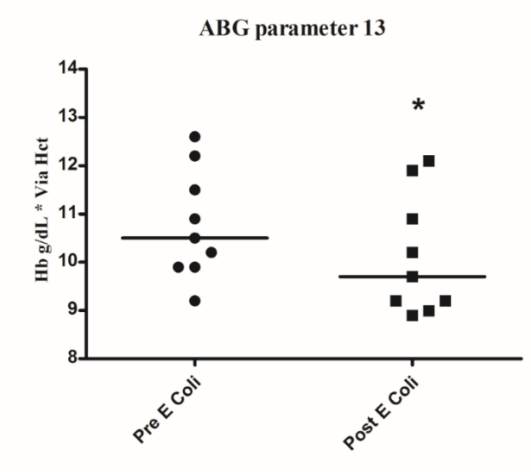


Figure 30. Hemoglobin before and after sepsis

Hemoglobin value decreased from 10.88 ± 1.13 to 10.23 ± 1.22 after administration of *Escherichia Coli* with a p-value of **0.005** which is significant statistically with WSR test (n=10).

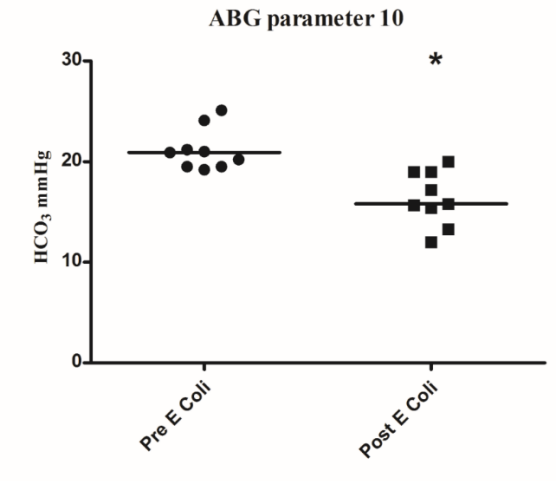


Figure 31. Serum bicarbonate before and after sepsis

Serum bicarbonate decreased from 20.92 ± 2.13 to 16.23 ± 2.58 after *Eschericia Coli* administration with a p-value of **0.005** which is statistically significant with WSR test (n=10).

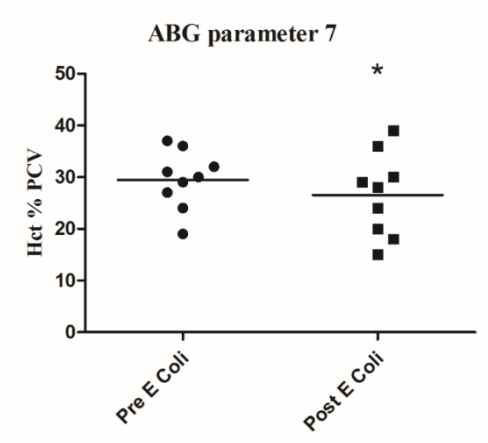


Figure 32. Hematocrit before and after sepsis

Hematocrit value decreased from 29.90 ± 5.50 to 27.10 ±7.80 after administration of *Escherichia Coli* with a p-value of **0.032** which is also significant statistically with WSR (n=10).

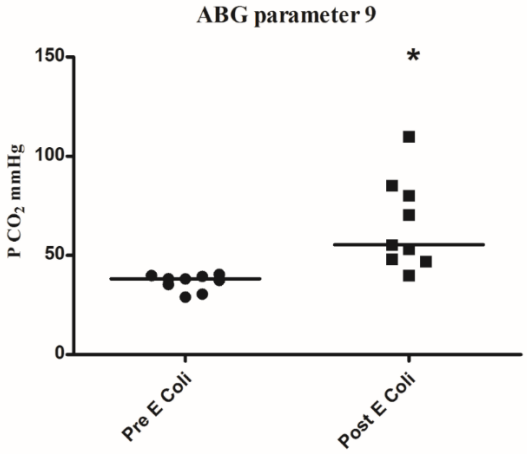


Figure 33. P CO_2 before and after sepsis

P CO_2 increased from 36.09 ± 4.08 to 65.35 ± 21.50 after administration of *Escherichia Coli* with a p-value of **0.005** which is significant statistically with WSR test (n=10).

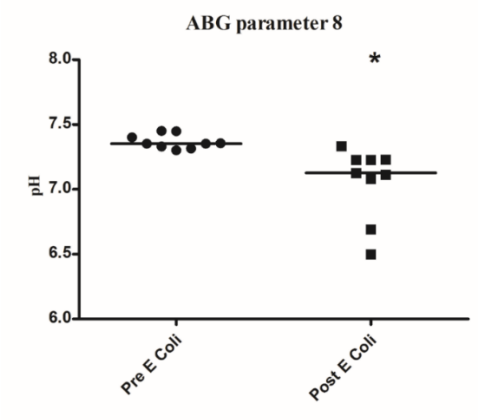


Figure 34. Blood pH before and after sepsis

Blood pH decreased from 7.37 ± 0.55 to 7.051 ± 0.26 after administration of *Escherichia Coli* with a p-value of **0.005** which is statistically significant with WSR test (n=10).

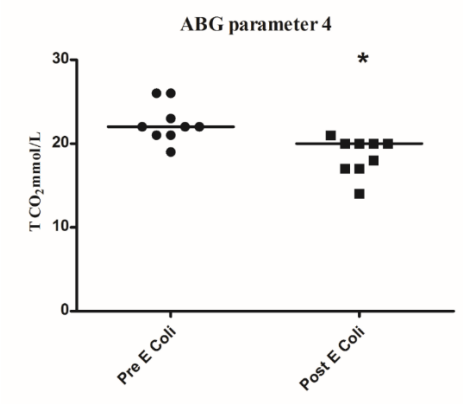


Figure 35. Total CO_2 before and after sepsis

Total CO2 value decreased from 22.20 ± 2.30 to 18.50 ± 2.12 after administration of *Escherichia Coli* with a p-value of **0.005** which is also significant statistically with WSR test (n=10).

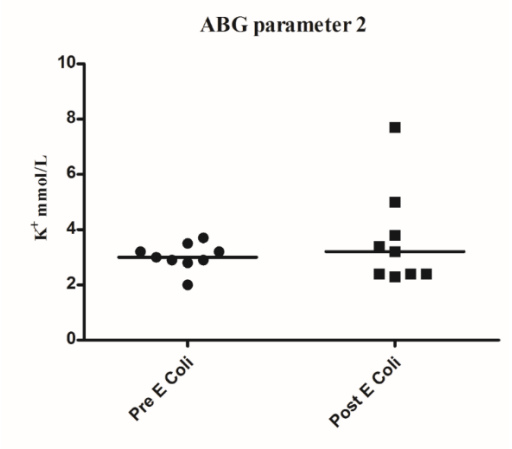


Figure 36. Serum potassium before and after sepsis

Serum potassium values increased from 3.03 ± 0.46 to 3.65 ± 1.67 after administration with *Escherichia Coli* with a p-value of 0.573 which is not significant statistically with WSR test (n=10).

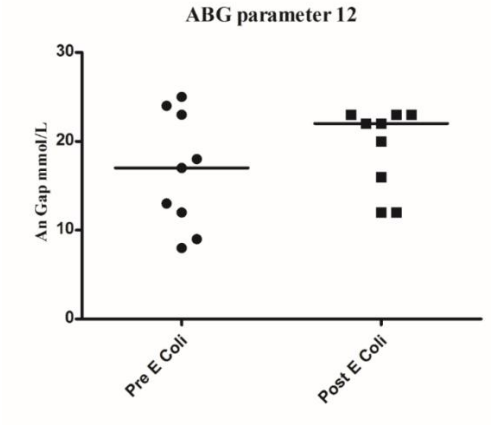


Figure 37. Anion Gap before and after sepsis

Anion Gap increased from 17.30 ± 6.53 to 19.30 ± 4.39 after administration of *Escherichia Coli* with a p-value of 0.212 which is not significant statistically with WSR test (n=10).

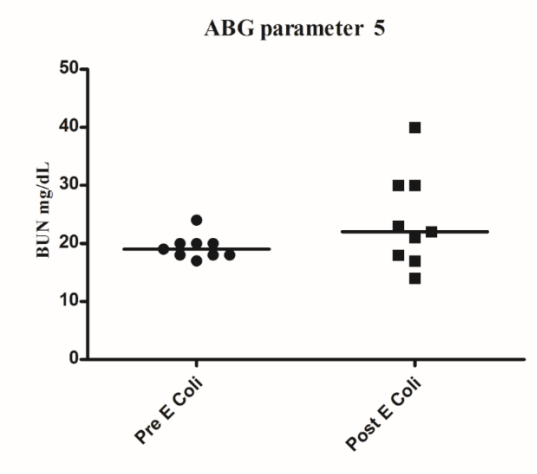


Figure 38. Blood Urea Nitrogen before and after sepsis

BUN values increased from 19.10 ± 2.07 to 25.70 ± 9.55 after administration of *Escherichia Coli* with a p-value of 0.108 which is not significant statistically with WSR test (n=10).

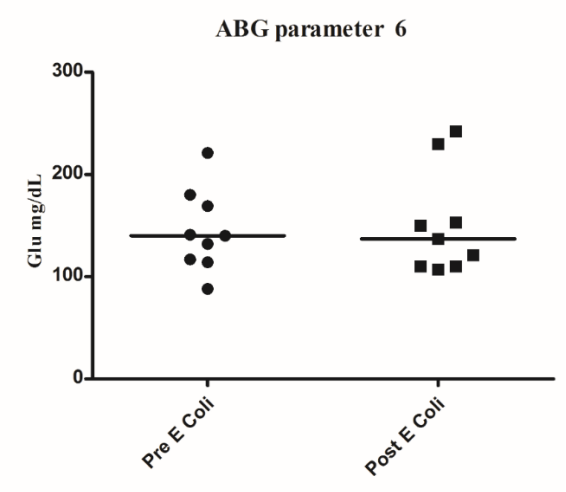


Figure 39. Serum Blood Glucose before and after sepsis

Serum blood glucose levels increased from 143.00 ± 38.05 to 148.00 ± 49.20 after administration of *Escherichia Coli* with a p-value of 0.878 which is not significant statistically with WSR test (n=10).

10. DISCUSSION

New Zealand (NWZR) female gender with a weight of 2733.60 ± 761.00 grams were effectively used to develop an animal model for septic shock using freshly isolated strains of *Escherichia Coli*.

Many studies were done on rabbits to establish an animal model for sepsis and septic related studies. Several studies utilized an invasive surgical method to induce sepsis either by creating a perforation in the bowel or by injecting the components of bacteria such as lipopolysaccharide or synthetically derived bacterial wall components. The time duration for establishment of sepsis varied widely in most of the studies ranging from 48 hours to 72 hours.

Our study is peculiar that the onset of sepsis were within minutes and the entire animal septic shock model were established within 136.80 ± 22.190 minutes. Rather using synthetically derived bacterial components or surgical methods to induce sepsis we developed sepsis by intravenously injecting the most common causative agent *Escherichia Coli* directly from the patients who suffered from sepsis related morbidities.

Drop in the Mean arterial pressure, Pulse pressure from the baseline recording ensured the development of septic shock leading to death. Intervention with an alpha agonist typically increased the Mean arterial pressure and pulse pressure delaying the death of the animal. Whereas the group of animals which received an alpha antagonist along with the additive effect of the bacterial strains hastened death.

Our study demonstrates an unexplained agonist activity of Phentolamine on the rabbit vasculature, which is actually an alpha antagonist.

Our study also demonstrates a slight reduction in the body temperature of the animal after the development of sepsis contrary to hyperthermia in sepsis. Arterial blood gas analysis reveals hypernatremia which can be related with the loss of fluid in the third space due to sepsis related vessel injury. Another striking finding in ABG is the development of combined Metabolic and Respiratory acidosis with the establishment of sepsis. The metabolic acidosis was associated with significant Hyperchloraemic acidosis. Anion gap did not show a significant difference though elevated slightly. Death in all the animals followed a similar pattern which is hypotension followed by reflex tachycardia, Respiratory distress with reflex tachypnea. Failure of the compensatory mechanisms finally led to circulatory collapse and death of the animal.

11. CONCLUSIONS

1. Effective Rabbit model of Escherichia *Coli* septic shock is developed to study the pathophysiology of septic shock related deaths.

2. Response to known intervention were as expected

    a. Alpha agonist increases MAP thus delaying death in septic shock.

    b. Alpha antagonist along with the additive effect of *Escherichia Coli* hastened death in septic shock.

3. The model can be extended using other live strains of bacteria.

12. LIMITATIONS

Other parameters of sepsis like C - reactive protein, Total Leucocyte Count, Coagulation profile and post sepsis *Escherichia Coli* count was beyond the scope of this study which could have been done.

13. FUTURE PROSPECTS

1. We are trying to establish other models of animal septic shock using alternative causative agents as well as fungi.
2. Firstly we are eyeing on *Klebsiella pneumoniae* since there is an alarming rise in the sepsis caused by these agents.

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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. Aravindhan V,
PG Registrar,
Department of Physiology,
Christian Medical College,
Vellore 632 002.

Sub: **Fluid Research Grant: New Proposal:**
Rabbit model for septic shock using bacterial strain isolated from patients with sepsis.
Dr. Aravindhan V (Emp. No. 21549), PG Registrar, Physiology, Dr. Sathya Subramani (Emp. No. 14123), Physiology, Dr. Sajo Thomas (emp. No. 21470), Physiology.

Ref: IRB Min. No. 11032 [OTHER] dated 04.12.2017

Dear Dr. Aravindhan V,

I enclose the following documents:-

1. Institutional Review Board approval 2. Agreement

Could you please sign the agreement and send it to Dr. Biju George, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

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.

Cc: Dr. Sathya Subramani, Dept. of Physiology, CMC, Vellore

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294, 2284202 Fax: 0416 – 2262788, 2264481 E-mail: research@cmcvellore.ac.in

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

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Ref: IRB Min. No. 11032 [OTHER] dated 04.12.2017

Dear Dr. Aravindhan V,

The Institutional Review Board **(Blue**, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project titled "Rabbit model for septic shock using bacterial strain isolated from patients with sepsis" 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.

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294, 2284202 Fax: 0416 – 2262788, 2284481 E-mail: research@cmcvellore.ac.in

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

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
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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: "Rabbit model for septic shock using bacterial strain isolated from patients with sepsis" 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 Ist 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. 11032 [OTHER] dated 04.12.2017

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INSTITUTIONAL ANIMAL ETHICS CLEARANCE

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. Aravindhan V,
PG Registrar,
Department of Physiology,
CMC, Vellore.

Dear Dr. Aravindhan,

Your research proposal titled "Rabbit model for septic shock using bacterial strain isolated from patients with sepsis" has been reviewed by the Institutional Animal Ethics Committee (IAEC) at the meeting held on 26.02.2018.

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

The IAEC approval number for the study is **7/2018.**

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

Dr. A. Yasotha
CPCSEA – Main Nominee