ISSN: 2573-9611

Research Article

Journal of Pediatrics & Neonatal Biology

Development and Optimization of a Neonatal Rat Model of Sepsis

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Submitted: 18 Apr 2018; **Accepted**: 25 Apr 2018; **Published**: 30 Apr 2018

Abstract

*Neonatal sepsis is the most common cause of mortality in newborns. Currently antibiotics and supportive care are the mainstay of treatment. Blood culture is considered as the gold standard for confirmation of diagnosis of neonatal sepsis. Here we have tried to develop a neonatal rat model of sepsis in order to better understand its progression. Lipopolysaccharide (LPS) is one of the common agents used to induce sepsis in rats. Here we found that LPS was ineffective in inducing sepsis in neonatal rats. We found that induction of live dose of Escherichia coli, one of the most common causes of neonatal sepsis was more effective than LPS injection. The rats were continuously monitored for the visual indications of sepsis development. Body weight, body temperature and the activity of rats were monitored continuously. Blood culture was done to check for the confirmation of diagnosis of sepsis. Further biochemical tests such as citrate, urease, indole and kliger-ion tests were done to confirm for E coli in the colonies of blood culture. The minimum effective dose of E coli needed to induce sepsis in neonatal rats was found to be 5*10^6 CFU of E coli.*

Keywords: Neonatal Sepsis, Blood culture, Lipopolysaccharide, Escherichia Coli, Diagnosis

Introduction

Sepsis is one of the common cause of neonatal mortality accounting for 30 to 50% deaths in the developing countries [1,2]. The incidence of neonatal sepsis according to the National neonatal and perinatal database (NNPD 2002-03) is 30 per 1000 live births. This database comprised of 18 tertiary care neonatal units across India and it showed that neonatal sepsis accounts for 19% of all neonatal deaths [3]. The earliest signs of neonatal sepsis are non-specific which makes diagnosis and treatment difficult. The common signs include fever or hypothermia, lethargy, poor feeding, respiratory distress, apnea, hypo/hyperglycemia and metabolic acidosis [4]. Blood culture is considered as the gold standard for confirmation of the diagnosis of neonatal sepsis [5,6]. A positive blood culture with sensitivity to the suspected causative organism is the best indicator to the type of antimicrobial therapy which has to be initiated. Animal models of sepsis have helped in the better understanding of the syndrome. Lipopolysaccharide (LPS) has been used for studying the inflammation related mechanisms and cytokine profiles in human endotoxemia models [7]. Bacterial administration to animals has been used to study the infection related host response mechanisms [8]. *Escherichia coli* (*E coli*) has been commonly used to induce infection in animals and to study the mechanisms of sepsis. The infusion with *E coli* causes rise in pro-inflammatory cytokines which cause severe tissue and organ damage [9]. Here we have attempted to the study the response of neonatal rats to LPS and *E coli*. The minimum optimal dose of *E coli* which is needed to induce and study sepsis in neonatal rats has been found by trying different doses of *E coli*.

Material and Methods

Experimental Animals

This study protocol was reviewed and approved by the Institutional Animal Ethics Committee (IAEC 21/6/2015). Wistar albino rats aged within 10 days after birth and 10-15g in weight were procured from the Animal House. The animals were randomly divided into four groups comprising of five animals (n=5) in each group. The neonatal rats along with their mothers were maintained at 18-25'C in a 12h dark-light cycle. The rats were kept in standard animal cages and fed with rodent chow and UV sterilized water.

Chemicals

Lipopolysachharide (LPS) was purchased from Santa Cruz Biotechnology and dissolved in sterile distilled water (5 mg/ml). Chemicals for biochemical assays such as citrate, urease, indole and kliger-iron agar were purchased from HiMedia Labs.

Preparation of *E coli*

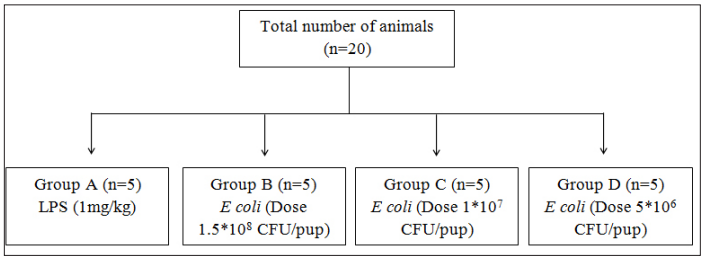
Escherichia coli (ATCC 25922) culture was received from Department of Microbiology, JIPMER in brain and heart infusion stock (BHI). It was subcultured and incubated for 24h at 37'C. The optical density was checked at 600nm using spectrophotometer. An optical density of 1 corresponds to 5x10^8 to 1x10^9 cells/ml. Accordingly three different concentrations of *E coli* was prepared - 1.5*10^8 cells/ml, 1*10^7 cells/ml and 5*10^6 cells/ml. After 24 h, the LB broth containing *E coli* was centrifuged and the pellet was collected

and suspended in saline. The turbidity of the *E coli* cells suspension was compared with the Mac-Farland standard set (Himedia Labs).

Development and optimization of neonatal sepsis rat model

Rat pups of age less than 10 days are selected from different parent rats. The rats were divided into 4 groups of 5 animals each. The body weight of all the rats was recorded before and after infection/ treatment. First group of rats Group A (n=5) were injected with intraperitoneal dose of LPS (1mg/kg). The next 3 group pups are infected by intraperitoneal injection with three different concentrations of *E coli* suspended in saline: Group B ($1.5*10^8$ CFU), Group C ($1*10^7$ CFU) and Group D ($5*10^6$ CFU) at 1mg/ kg bodyweight.

Within few hours of *E coli infection*, animals were observed for clinical signs of sepsis that includes piloerection, lethargy, skin colour changes (pale red), huddling and a decrease in food intake [10]. The activity of the rats was monitored using the activity meter (Coulborn Instruments, USA). Rectal temperature was measured periodically to observe for the fluctuations in body temperature. Blood culture was done from the blood collected from all the rats and the plates were continuously observed for colony growth for the next 72 hours. Further biochemical tests (citrate, indole, urease, kligers-iron agar) were done to confirm for *E coli* in the culture. Mortality and symptoms was monitored continuously.



Results

Activity of Rats

The neonatal rats in the various study groups were observed for clinical signs of sepsis such as piloerection, lethargy, reduced food intake, water intake. The food intake and activity of the rats were checked in the activity meter. The results observed were as follows:

Group	Food intake (g)	Drink (g)	Activity
Group A (LPS)	55.2	62.2	104
E coli Group B	9.6	7.5	7
E coli Group C	12.5	18.5	12
E coli Group D	32.5	36.2	32

Body Temperature

The body temperature was recorded using a rectal thermometer. The results are as follows:

Group	Average Body Temperature (degree Celsius)		
	Before Sepsis	1 hour after Sepsis	6 hour after Sepsis
Group A (LPS)	36.0 ' C	36.3' C	36.2 ' C
E coli Group B	36.1 ' C	38.7 ' C	40.7 ' C
E coli Group C	37.0' C	37.4' C	39.9' C
E coli Group D	36.9' C	37.2' C	38.1' C

Mortality

Group	Survival percentage (%)			
	1h post sepsis	3 h post sepsis	6 h post sepsis	12 h post sepsis
Group A (LPS)	100%	100%	100%	100%
E coli Group B	40%	20%	0%	0%
E coli Group C	60%	40%	20%	0%
E coli Group D	100%	100%	80%	60%

Blood culture

Plates streaked with blood samples from individual rats from all the groups were incubated for 24h at 37'C. The 3 groups of rats induced with *E coli* showed significant growth (Figure 1, b-d)

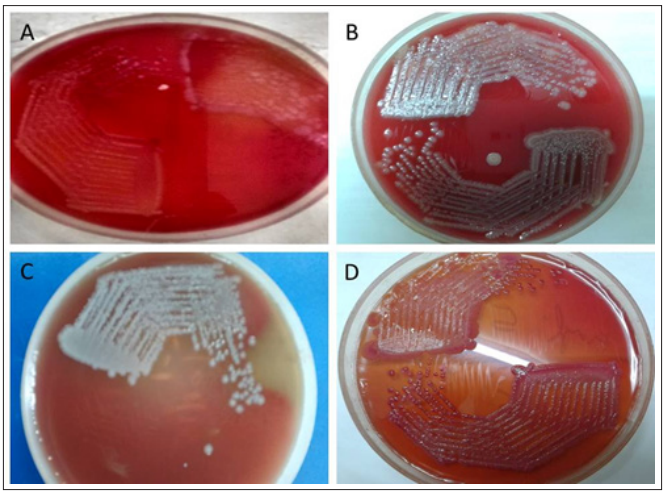


Figure 1: Blood culture results. A-Representative images from Group A ; B-Group B; C- Group C; D- Group D

Biochemical Tests

Biochemical tests were done for confirmation of *E coli* infection. Biochemical tests confirmed the presence of *E coli* in plates of Groups B, C, D. (Fig 2)

1. **Citrate Test**: All samples of *E coli* group (B,C,D) showed citrate negativity, thereby confirming organism as *E coli*.
2. **Indole Test**: All the samples of *E coli* group (B,C,D) were confirmed to be indole positive.
3. **Urease Test**: *E coli* is urease negative organism which was evident in all samples of *E coli* sepsis group.
4. **Kliger's Iron Agar (KIA)**: In all the samples of *E coli* sepsis group, the organisms were capable of utilizing glucose and lactose and thus it remained yellow. Gas production was observed (A+) but no H2S production (no black shade at the bottom of tube) was seen (A+/A-). This further confirms *E coli* infection.

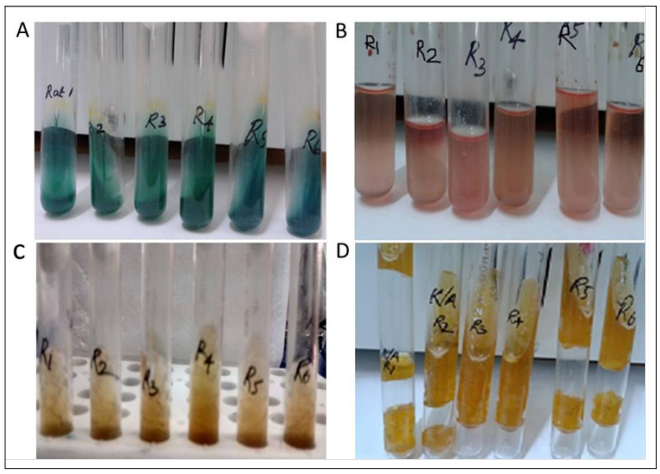


Figure 2: **Biochemical tests confirmed the presence of *E coli*.** A-Citrate test; B- Indole test; C- Urease test; D- Kliger-irons test

Discussion

Out of the 130 million babies born every year, 4 million babies die within the first 4 weeks of life [11]. This period accounts for 38% of all deaths in the children younger than 5 years of age. Severe infections including sepsis account for 36% of the direct causes of neonatal deaths [12]. Despite the advancement in medical science, neonatal sepsis still accounts for high morbidity and mortality among term and preterm newborns [13]. This is mostly attributed to the non-specific signs and ineffective treatment [14]. Antibiotics and supportive care are the mainstay of treatment and increasingly resistant forms of microbes are found nowadays. Animal models of sepsis have been used to understand the pathophysiology of sepsis. In this study we have tried to establish the development of neonatal sepsis in rat models using LPS and *Escherichia coli* as the source of infection.

Endotoxins such as lipopolysaccharide (LPS) have been used for the induction of sepsis. LPS has been used for studying the inflammation related mechanisms and cytokine profiles in human endotoxemia models [7]. The injection of LPS to human volunteers causes symptoms like fever, headache, increase in body temperature and other mild effects, which fade after few hours [15,16]. But compared to humans, experimental animals seem comparatively insensitive to LPS and require almost 250 times higher dose to induce similar cytokine response [17]. The same study also showed that the administration of endotoxin induced a rampant physiological response such as fever and tachycardia in humans but not in mice. In this study also it was found that LPS (5 mg/ml) induction to neonatal rats (1mg/kg) produced no aberrant physiological changes. The rats were quite normal after LPS injection and showed no signs or symptoms of sepsis development for 12 hours. The activity, feeding intake and body temperature values were normal throughout the study period. The survival was 100% at 12 hours after injection of LPS.

Gram negative pathogens are the major causative agents for neonatal infection. *Escherichia coli is* one of the most predominant bacterial pathogen found in community acquired infections in various hospitals [18]. *E coli* are the most common causative agents of early onset neonatal sepsis, and accounts for high mortality in neonatal sepsis [19]. In our study we tried to optimise the dose of *E coli* needed to study the pathophysiology of neonatal sepsis. The highest dose of *E coli* used was $1.5*10^8$ CFU/pup (Group B). This dose was found to be very severe and the mortality was 100% in just over 3 hours of induction of sepsis. There was very rampant rise of body temperature and much reduced activity and food intake. The Group C rats were induced with $1*10^7$ CFU of *E coli*. This group showed similar physiological signs of sepsis but the intensity was slightly lower than Group A. The mortality was 100% in just over 6 hours. The Group D rats were induced with $5*10^6$ CFU/pup. This dose was found to be ideal for studying the features of neonatal sepsis rats. There was a gradual increase of body temperature over 12 hours. There was also a steady decrease of activity and feeding intake. The mortality was 60% at 12 hours post sepsis which suggests that this dose could be used for the optimal development of neonatal sepsis in rats.

Blood culture is considered as the gold standard for the diagnosis of neonatal bacterial sepsis [5,6]. In this study blood was collected from all the rats immediately after death for blood culture and the plates were continuously observed for colony growth for the next 72 hours. We used this for confirming bacterial sepsis. The sepsis group rats showed growth of colonies within 24 hours of plating, unlike the Group A which did not show any growth (Fig 1A). In order to confirm the organism in the colony, further standard biochemical tests were done and it was confirmed that the source of infection was *Escherichia coli*.

Conclusion

This study has shown that infusion of *E coli* in neonatal rats is a better model than the injection of LPS. The neonatal rats were insensitive to the dose of LPS. Moreover the minimum effective dose of *E coli* needed to develop neonatal sepsis in rats was found to be $5*10^6$ CFU/pup.

Acknowledgement

We would like to thank the members of the Department of Neonatology, Microbiology and Pathology, JIPMER for their continuous support throughout the course of this study.

Funding

JIPMER Intramural Research Fund

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Ethical Approval

This study has been approved by the JIPMER Institutional Animal Ethics Committee (IAEC).

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