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# VALIDATION OF A MURINIC MODEL OF SEPSIS INDUCED BY CECAL LIGATION AND PUNCTURE TECHNIQUE

**M. MĂRGINEAN[1]**
**A. ARDELEAN-MAGHIAR (MĂRGINEAN)[2]**
**F. ŞULAR[3] D. DOBREANU[4]**

***Abstract:*** *Sepsis is considered to be a systemic response to an infection. This pathology is an common cause for mortality and morbidity being the primary cause of death among patients in the Intensive Care Units. Because of the increasing need for a standardised animal model of sepsis we decided to validate a murinic one using the cecal ligation and puncture (CLP) technique and easy to obtain physiologic and laboratory parameters.*

***Key words:*** *sepsis, cecal ligation and puncture, CLP, rat, animal model.*

## 1. Introduction

Sepsis was defined as a systemic inflammatory response syndrome to an infectious injury [1] [9]. This pathology is increasingly common in the Intensive Care Units (ICU) where it is responsible for the majority of deaths [11][12]. Until recently it was agreed upon that even if the syndrome itself is triggered by an infectious injury, the pathophysiology of sepsis is determined by an exaggerated hyperimmune response and the formation and presence of a number of pro-inflammatory agents like cytokines, chemokines and complement activation factors that will ultimately prove to have a destructive effect – uncontrolled inflammatory response theory [10]. Even if this theory was not abandoned altogether, new experimental evidence has shown that the immune reaction of the human body is far more complex than previously thought and the multiple organ and system disfunctions encountered in sepsis are in fact the result of an immune imbalance and the lack of coordination between the anti-inflammatory and pro-inflammatory factors that translate in an initial hyper-immune state followed by an immunological paralysis [5], [14]. During this later phase the highest mortality is recorded.

Because of the high incidence and severity of this syndrome, extensive research has been conducted to study the etiology, conventional treatment response and novel therapeutical targets in sepsis. For these studies researchers have developed different animal models. Due to its clinical

[1] *Anaesthesia and Intensive Care*, Emergency Military Hospital, Cluj-Napoca, Romania | www.smucluj.ro.
[2] *Laboratory*, Emergency Military Hospital, Cluj-Napoca, Romania | www.smucluj.ro.
[3] *Central Laboratory*, Emergency County Hospital, Târgu-Mureş, Romania | www.spitjudms.ro.
[4] Physiology Department, University of Medicine and Pharmacy, Târgu-Mureş, Romania | www.umftgm.ro.

resemblance to human sepsis, the most widely used is the cecal ligation and puncture model which consists of the perforation of the cecum allowing the release of faecal material into the peritoneal cavity. This will generate an immunological exacerbation in response to polymicrobial blood stream infection.

## 2. Objectives

The purpose of this study was to validate a model of sepsis using rats as experimental animals and easy to obtain and measure parameters with low as possible financial burdening.

## 3. Methods

For this study we used 14 male Wistar rats obtained from the Experimental Station of the *University of Medicine and Pharmacy* in Târgu-Mureş. They were housed and fed under standard conditions with controlled temperature and a 12 hour imposed sleep-wake cycle. The experiment was performed with regard to the 2010 *Directive of the European Union* [2] and after the approval of the Ethics Committee for scientific research. The animals were randomised in two groups: control (C, *n=6*) and with sepsis (SEP, *n=8*). Anaesthesia was obtained using intraperitoneal Ketamin 100 μg/g and Xylazine 10 μg/g. At the incision site fur was removed and sterile operating field obtained. For the control group we performed a *sham* operation consisting in the abdominal wall incision, entering the peritoneal cavity, extracting the cecum, placing the cecum back inside the peritoneal cavity, closing the peritoneum with absorbable sutures and the abdominal wall with non-absorbable ones. For the SEP rats we additionally performed the *cecal ligature and puncture* (CLP) procedure [6], [7] consisting of the ligation of cecum at 1 cm from its distal end using a non-absorbable suture. We then placed two punctures on the same side of the cecum using a 19G needle. A small amount of faeces was extruded by gently squeezing the cecum to confirm penetration. The cecum was then returned to the peritoneal cavity and anatomical layers were sutured as described earlier [17]. Fluid resuscitation was achieved by subcutaneously injecting of 0.05 ml/g prewarmed normal saline solution using a 23G needle [13]. This fluid resuscitation measure will induce the hyperdynamic phase of sepsis. During the entire surgical intervention animals were kept under warm light and body temperature was monitored using a rectal probe. Normal body temperature was also ensured until full reversal of anaesthesia. Fluid administration was repeated every 24 hours. Postoperative analgesia was obtained by subcutaneously injecting of Tramadol (20 μg/g) on daily basis or whenever it was needed according to specific pain scales [3], [16] . After the surgical procedure the rat's tail was warmed under 40° water and blood was obtained by puncturing lateral tail veins. A 23G needle was used after flushing with Heparin Sodium (5000 IU/ml) to prevent blood clotting inside the needle. We did not use a syringe to draw blood but allowed the blood to freely drip inside the standard $K_2$ EDTA microtainer avoiding possible haemolysis due to aggressive aspiration. Blood drawing was repeated in day 4, day 7 and day 14. Every time a complete blood count was obtained using a *Sysmex Hematology System.* Each time the rat was anaesthetised as described earlier. Body mass was also assessed at the same intervals (day 1, day 4, day 7 and day 14).

## 4. Results

All parameters from the complete blood

count and body mass measurements were imported into a *Microsoft Excel* worksheet. *Grubbs* test was performed and no outliers were found. Descriptive statistics was obtained using *Microsoft Analysis ToolPak* (Tables 1 and 2).

*Descriptive statistics. Body mass* Table 1

| | **C-1** | **C-2** | **C-3** | **C-4** | **SEP-1** | **SEP-2** | **SEP-3** | **SEP-4** |
|---|---|---|---|---|---|---|---|---|
| Mean | 364.5 | 354.17 | 354.5 | 335.83 | 410.75 | 379.13 | 372 | 365.25 |
| Standard Error | 26.2 | 26.07 | 25.59 | 16.72 | 21.32 | 15.46 | 14.42 | 19.82 |
| Median | 359 | 336 | 334 | 325.5 | 405.5 | 380 | 378 | 372 |
| Mode | #N/A | #N/A | #N/A | #N/A | #N/A | #N/A | 378 | #N/A |
| Standard Deviation | 64.18 | 63.87 | 62.69 | 40.97 | 60.31 | 43.74 | 40.8 | 56.06 |
| Sample Variance | 4119.50 | 4078.97 | 3929.5 | 1678.17 | 3637.07 | 1912.98 | 1664.29 | 3142.21 |
| Kurtosis | -1.25 | -1.12 | -1.30 | 3.16 | 2.25 | 0.4 | 0.97 | 2.5 |
| Skewness | 0.21 | 0.70 | 0.76 | 1.69 | 1.20 | 0.00 | -0.07 | 0.06 |
| Range | 168 | 163 | 153 | 113 | 198 | 143 | 137 | 203 |
| Minimum | 284.00 | 287.00 | 295.00 | 300.00 | 337.00 | 309.00 | 305.00 | 265 |
| Maximum | 452.00 | 450.00 | 448.00 | 413.00 | 535.00 | 452.00 | 442.00 | 468 |
| Sum | 2187 | 2125 | 2127 | 2015 | 3,286 | 3033 | 2976 | 2922 |
| Count | 6.00 | 6.00 | 6.00 | 6.00 | 8.00 | 8.00 | 8.00 | 8.00 |

*Descriptive statistics. WBC count* Table 2

| | **C-1** | **C-2** | **C-3** | **C-4** | **SEP-1** | **SEP-2** | **SEP-3** | **SEP-4** |
|---|---|---|---|---|---|---|---|---|
| *Mean* | 7.02 | 8.90 | 7.37 | 6.28 | 9.60 | 12.79 | 11.25 | 8.25 |
| *Standard Error* | 0.42 | 1.10 | 0.17 | 0.76 | 0.74 | 0.67 | 0.71 | 0.83 |
| *Median* | 6.95 | 8.70 | 7.40 | 6.75 | 9.50 | 13.25 | 10.85 | 7.45 |
| *Mode* | 7.50 | #N/A | #N/A | #N/A | #N/A | #N/A | 9.70 | 7.40 |
| *Standard Deviation* | 1.02 | 2.70 | 0.41 | 1.87 | 2.10 | 1.90 | 2.01 | 2.35 |
| *Sample Variance* | 1.05 | 7.28 | 0.17 | 3.50 | 4.40 | 3.60 | 4.04 | 5.53 |
| *Kurtosis* | -0.93 | -1.06 | 0.87 | 1.33 | -0.39 | -1.26 | 1.97 | 0.24 |
| *Skewness* | 0.60 | 0.52 | -0.59 | -0.90 | 0.67 | -0.48 | 1.33 | 0.81 |
| *Range* | 2.60 | 6.80 | 1.20 | 5.50 | 6.10 | 5.10 | 6.20 | 7.30 |
| *Minimum* | 6.00 | 6.20 | 6.70 | 3.10 | 7.10 | 9.90 | 9.20 | 5.00 |
| *Maximum* | 8.60 | 13.00 | 7.90 | 8.60 | 13.20 | 15.00 | 15.40 | 12.30 |
| *Sum* | 42.10 | 53.40 | 44.20 | 37.70 | 76.80 | 102.30 | 90.00 | 66.00 |
| *Count* | 6.00 | 6.00 | 6.00 | 6.00 | 8.00 | 8.00 | 8.00 | 8.00 |

The inferential statistics was performed using *GraphPad InStat* version 3.05 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. To compare means, paired *t* test was employed with the Kolmogorov-Smirnov normality test. Statistical significance is considered if a *p* value under 0.05 is obtained.

For body mass, we compared the values obtained in the first day (C1/SEP1) with those from the following measurements - day 4 (C2/SEP2), day 7 (C3/SEP3) and day 14(C4/SEP4). There was no difference between C1 and C2 *(p*=0.15*)*, C1 and C3 *(p*=0.21*)* or C1 and C4 *(p*=0.23*)* regarding body mass. When comparing SEP1 with SEP2 (Fig.1) and with SEP3 (Fig.2) we found a statistical difference with a $p$=0.01 in both. SEP1 and SEP4 differed with a value considered very significant *(p*=0.003*)* (Fig.3). The median was consistently higher in the SEP1 data set (Table 1).

From the complete blood count panel only WBC count showed statistical differences. Again, when comparing the values from the control group we did not find any differences (C1-C2 showed a $p$=0.15*;* C1-C3 $p$=0.5; C1-C4 $p$=0.43). In the septic group we found a strong difference between SEP1 and SEP2 (*p=0.003*) (Fig. 4) with the highest median in the SEP2 data set (Table 2).

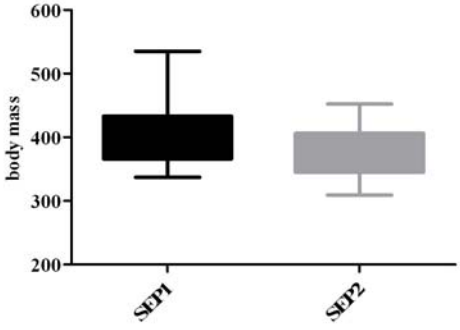


Fig. 1. *Body mass. SEP1-SEP2*

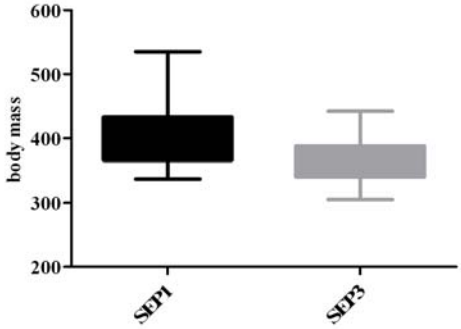


Fig. 2. *Body mass. SEP1-SEP3*

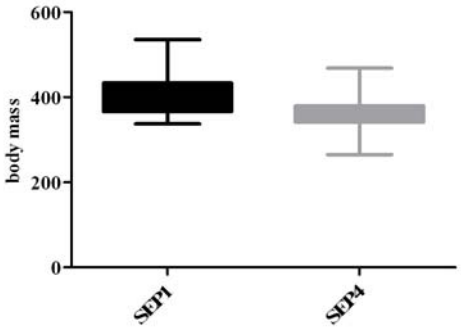


Fig. 3. *Body mass. SEP1-SEP4*

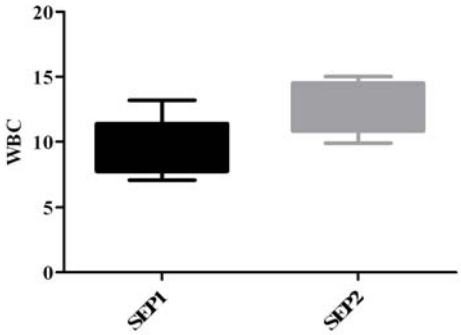


Fig. 4. *White blood cell count. SEP1-SEP2*

There was also a small difference between the SEP1 and SEP3 data sets but with a value of $p$ considered non-significant ($p$=0.07) (Fig.5). SEP1 and SEP4 showed no difference ($p$=0.13) (Fig.6, Fig.7).

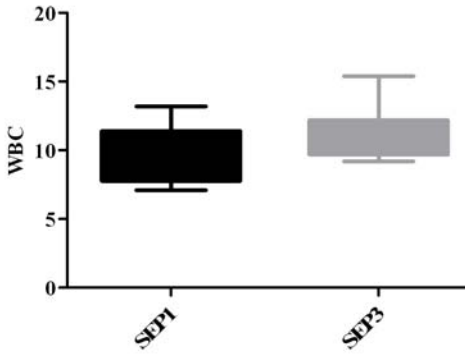


Fig. 5. *White blood cell count. SEP1-SEP3*

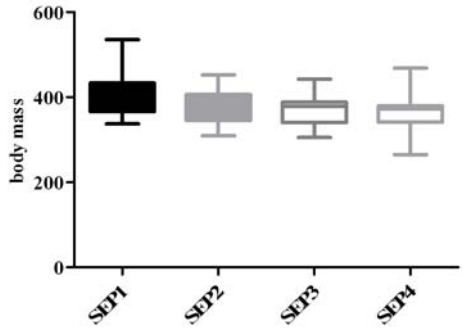


Fig. 6. *Body mass. Entire SEP data set*

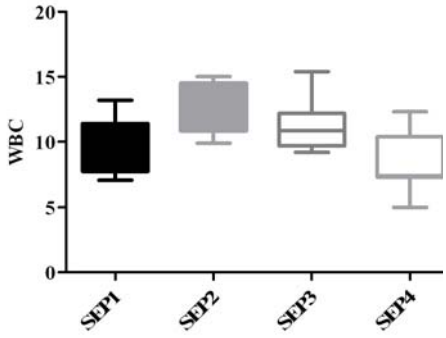


Fig. 7. *White blood cell count. Entire SEP data set*

## 5. Discussions

This model once validated will assure the basis for further research involving sepsis. We consider that the strength of this sepsis model resides in its simplicity, repeatability and relative lack of financial burdening. The CLP procedure has been accused in the past that despite of its obvious advantages and the fact that it induces a form of sepsis that resembles the one found in humans, the lack of method standardisation made results difficult to reproduce [15]. This shortcoming was recently overtaken thanks to the efforts of Toscano *et al*. who published considerable enhancements of the classic CLP technique, including the relation between the length of the excluded cecal segment and the intensity of the septic syndrome or the correlation between septic severity and the number of cecal punctures [17].

As an improvement of the presented model we propose the use of C-Reactive Protein (CRP) blood measurements and erythrocyte sedimentation rate to prove blood stream bacterial presence as previously reported [18] as well as determining serum Procalcitonin (PCT) levels [4], [8].

## 6. Conclusion

We conclude that CLP technique is a viable method to induce sepsis in a murinic model and that by monitoring body mass and white blood cell count this model could be validated.

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