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Chapter 2

Experimental Sepsis Models

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1. Introduction

Sepsis is a devastating condition characterized by the systemic activation of inflammatory and coagulation pathways in response to microbial infection of normally sterile parts of the body. Severe sepsis, defined as sepsis with at least one dysfunctional organ, is the leading cause of death in non-coronary intensive care units and is associated with mortality rates of 30-50%. The development of experimental sepsis models to elucidate the progression and pathophysiology of clinical sepsis spans the past eight decades. Studies utilizing models of intra-abdominal sepsis began in the 1930's with the isolation of endotoxin and the intravenous or peritoneal infusion of live organisms, a model which dominated sepsis research for over 30 years. In the 1960's, a transition was made from endotoxemia models to a focus on bacteremia. Such models include the injection of live bacteria (Pseudomonas aeruginosa, Escherichia coli, and Staphylococcus aureus), inoculation of feces, and intramuscular, intraperitoneal and subdermal implantation of feces-containing capsules and sponges. Following the use of endotoxemia and bacteremia models, various models involving ischemia and bowel perforation were developed. These models led to the development of the most frequently used sepsis model today, cecal ligation puncture (CLP) and more recently, the colon ascendens stent peritonitis (CASP) model. Over the years several large animal models of sepsis have also been developed, of which canine, ovine, bovine, rabbit, and non-human primates have proven to be most useful. However, the translation of findings and inferences from animal sepsis models to human sepsis remains a challenge. In this chapter, we will provide an overview of experimental models of sepsis, with focus on the merits and limitations of each model. We will also focus on strategies that may improve the translation of results from animal studies to human sepsis. This requires consideration of the limitations of current sepsis models including supportive therapies, considering age, gender, obesity, and exposure to pathogens in animals used, and addressing the risk of bias in experimental sepsis models.

2. History of experimental sepsis models

Sepsis is a serious, complicated, heterogeneous condition involving a dysregulated host response to an initial infection and subsequent hemodynamic, cardiovascular, respiratory, metabolic, hormonal, inflammatory, innate and adaptive immune changes (1;2). Experimental sepsis models have been utilized extensively and developed over the last eight decades to study the progression and pathophysiology of clinical sepsis (3-5). Intra-abdominal sepsis models were introduced in the 1930s with the isolation of endotoxins and the intravenous or peritoneal infusion of live organisms, models which dominated sepsis research for thirty years. Upon the discovery of new antimicrobial compounds, treatment of intra-abdominal abscesses became a clinical focus in the 1960s and a transition was made from the endotoxin model to the bacterial model of infection (4). These models used fecal pellets and the addition of an adjuvant to induce infection with the goal of studying peritonitis rather than isolated abscess formation (3). However, these models fell short as mortality rates relied heavily on bacterial counts and bacterial composition which varied widely between both species and individual subjects. Additionally, the bacterial overload at non-physiologically relevant levels and lack of a sterile environment contributed to a much greater mortality rate in experimental sepsis compared to what was observed clinically (4).

To overcome these limitations, the introduction of defined bacterial inoculum models in the 1970s improved sepsis modeling immensely. These models were also used in landmark studies which uncovered the two-stage nature of intra-abdominal sepsis with gram-negative enteric bacteria inducing the peritonitis phase and anaerobic bacteria responsible for subsequent abscess formation (3). Antibiotic agents tested using defined bacterial models were successful in either improving survival or reducing abscess formation but were not successful in improving both criteria. This led to the development of models which more closely resembled the progression clinical sepsis.

In the ‘60s and ‘70s, the Clowes group and Wright group were able to create septic conditions in dogs by ligating the cecum at the ileocecal valve, successfully demonstrating both the initial hyperdynamic phase and later hypodynamic phase of sepsis, but neither groups documented bacterial cultures to validate dissemination of pathogens in the peritoneum or circulation (6-9). A small animal model was later introduced by Ryan *et al* whereby the rat cecum is ligated distal to the ileocecal valve, devascularizing the cecum and introducing a necrotic component to experimental sepsis (10). It is this latter model which lead to the most frequently used animal model of sepsis, cecal ligation and puncture.

3. Common models of polymicrobial sepsis

3.1. Cecal Ligation and Puncture (CLP)

The CLP model of intra-abdominal sepsis was introduced by Wichterman, Baue, and Chaudry in 1980. The group published an insightful review of previous models and introduced a novel sepsis model still widely regarded as the gold standard for modelling polymicrobial sepsis today—the cecal ligation and puncture (CLP) model. Rats were fasted,

their cecum was ligated distal to the ileocecal valve, the antimesenteric cecal surface was punctured twice with an 18G ½ needle, and received subcutaneous saline post-operatively. This model induced polymicrobial infection (blood cultures positive for *Escherichia coli, Streptococcus bovis, Proteus mirabilis, Enterococcus,* and *Bacteroides fragilis)* and bacteremia (peritoneal cavity fluid positive for the above microbes as well as *Streptococcus viridians* and *Clostridium sporogenes)* and a 70% mortality rate. Mildly ill rats sacrificed 10 hours following CLP demonstrated the early hyperdynamic phase of sepsis (increased blood flow to organs, hyperinsulinemia, and hyperglycemia) while rats sacrificed 16-24 hours post-operative represented a hypodynamic late septic state (decreased blood flow to organs, hypoinsulinemia, hypoglycemia, and high serum lactate levels) (3). The results of this model correlate with clinical sepsis conditions as patients who are initially normotensive, show an increase in cardiac output, have low peripheral resistance, and increased total oxygen consumption, conditions which reverse in late septic shock (3).

Multiple aspects of the CLP procedure address the complex, of the clinical course of sepsis. CLP induces polymicrobial infection of the peritoneum with a localized infectious focus, release of bacteria and endotoxic molecular components of pathogens (pathogen-associated molecular patterns or PAMPs) into normally sterile areas in the host, and subsequent translocation of enteric bacteria into the bloodstream, modelling the stages of intra-abdominal clinical sepsis (3). Under anaesthesia, the cecum is exposed and trauma is induced via a midline skin laparotomy and blunt dissection of the peritoneum to exteriorize the cecum. Avoiding damage to the mesenteric vessels, the cecum is ligated with suture distal to the ileocecal valve, punctured once or twice (through-and-though) from the mesenteric to anti-mesenteric direction halfway between the ligation and cecal end, and aspirated for trapped gasses (3). A small amount of fecal content is extruded to allow for patency of the puncture(s) and continuous flow of feces post-operatively. The cecum is returned into the peritoneal cavity taking care not to spread fecal content on the incision and the peritoneum and abdomen are closed separately with sutures (11).

3.1.1. Host response to CLP

Significant elements of the host response to CLP-induced polymicrobial sepsis are present in clinical sepsis. The hemodynamic profiles, cardiovascular response, metabolic phases, systemic involvement of cytokine responses (ex. profiles of interleukins), changes in the innate and adaptive immune response, and abnormalities in mediators of coagulation which occur following CLP are also observed in the clinical course of sepsis (12). Moreover, CLP involves multiple, complicated elements which are unaccounted for by models of endotoxemia and bacterial inoculum. These include a laparotomy which mimics surgery-induced trauma in the septic patient, the presence of inflamed tissue (peritonitis), necrosis via ligation of the cecal end, apoptosis of specific leukocytes, bacteremia induced by pathogens from a host-derived flora (fecal spillage), and translocation of enteric, living, multiplying bacteria into the bloodstream (12). The inclusion of these elements as part of the CLP model improves the clinical relevance of outcomes from these preclinical studies.

As observed in clinical sepsis, the hyperinflammatory state which occurs during the systemic inflammatory response syndrome (SIRS) transitions to an immunosuppressed state characterized by a compensatory acute response (CARS) (12;13). However, the point at which this transition occurs in both clinical and experimental sepsis is unclear. The early hyperdynamic stage of sepsis and the later hypodynamic state following the CLP procedure is indicated by changes in response to immune challenge and changes in peripheral blood cells, plasma levels of cytokines, and chemokines. Neutropenia and lymphopenia are characterized by significant peripheral blood alterations rapidly following CLP parallel to leukocytosis or leukocytopenia observed in the clinical SIRS condition (1;12-14). Following CLP, total white blood cells, polymorphonuclear cells, and lymphocytes increase rapidly within the first 2 hours, decrease from 2-4 hours, and plateau until endpoint at 8 hours following CLP (15). The pro-inflammatory response is also characterized by significant increases in cytokines TNFα and IL-6 and chemokines KC, MIP-2, and MCP-1 from non-detectable plasma levels that increase and remain elevated over an 8 hour period (12;15). Several studies have demonstrated the importance of an early pro-inflammatory response in the progression of sepsis. Antibody-mediated blockade of IL-6 (16;17), complement factor C5a or C5a receptor (18-20), and depletion of neutrophils offered protective effects and increased survival of animals subjected to CLP.

While anti-inflammatory proteins like IL-10 and glucocorticoids are crucial for dampening and terminating the inflammatory response (21-23), the hypoinflammatory phase of experimental sepsis characterized by neutrophil paralysis (shutting down of signaling pathways), apoptosis of lymphocytes and dendritic cells, and elevations in anti-inflammatory mediators significantly increase the susceptibility of the septic host to nosocomial infection. It is during this hypoinflammatory, immunosuppressed state when most mortality is observed in clinical sepsis (21) however it is unknown if this holds true in animal sepsis.

3.1.2. Modifying severity in CLP

Multiple elements of the CLP procedure can be modified to model the wide spectrum of conditions observed in clinical sepsis. These factors include the number of cecal punctures, gauge of needle used to puncture the cecum, and the length of cecum ligated in the animal (3). However, there are conflicting findings as to whether the number of cecal punctures affects disease severity. One group reported that two cecal punctures does not result in a significant increase in mortality but is associated with a decrease time to endpoint (24). Variations in the CLP protocol can be used to produce different disease severities and mimic various stages of the sepsis spectrum from the rapid onset of a robust, hyperinflammatory state to a gradual progression of severe sepsis to an immunosuppression in septic shock. For instance, modifications such as using a smaller gauge/thicker needle (18G ½ rather than a 26G½) or ligating a larger amount of cecum (by placing the suture proximal to the ileocecal valve) can increase the severity and produce a mortality rate that may be more clinically relevant (25).

The flexibility in modeling various severities of disease, ability to recreate hemodynamic, metabolic, and immune changes, the inclusion of surgical trauma, necrosis, and apoptosis of specific cell types which more closely correlate with clinical sepsis contribute to the acceptance of CLP as the gold standard for modeling polymicrobial, intra-abdominal sepsis.

3.2. Colon Ascendens Stent Peritonitis (CASP)

Almost two decades following the introduction of the CLP model by Chaudry et al., Zantl et al. introduced a polymicrobial, peritonitis sepsis model termed colon ascendens stent peritonitis or CASP (26). The CASP model is a reproducible model suitable for studying the pathophysiology of abdominal sepsis and can be successfully varied by using stents of different diameters ranging from 14G to 22G (26;27). It can also be used to study surgical interventions which involve the elimination of the infectious focus by stent removal (27). The prevalence of the CASP model in sepsis studies has only recently increased. Currently, the number of studies utilizing the CLP sepsis model far exceed those using CASP (25).

In the CASP procedure, a laparotomy is performed to exteriorize the cecum, terminal ileum, and ascending colon. The ascending colon wall is pierced and the suture is fixed on the colon wall 15 mm distal from the ileocecal valve. A prepared stent or cannula is used to puncture the ascending colon around 1-2 mm proximal from the suture and the cannula is inserted into the colon and sutured securely. Fecal content is milked through the stent which provides a pathway between the intestinal lumen and the peritoneum, allowing unobstructed influx of enteric bacteria into the peritoneal cavity (26;28). Disease severity of this model, can be modified by adjusting the size of the cannula used for stenting from 14 G to 20 G (100% lethality and less than 50% mortality at 48 hours following surgery, respectively) (24;28). Within 3 hours of stent implantation, levels of circulating and systemic cytokines and chemokines including TNFα, IFN-γ, IL-1, IL-12, IL-18, KC/GRO-α, MCP-1, and anti-inflammatory IL-10 increase (26;29;30). Unlike CLP however, progression of sepsis in the CASP model appears less dependent on the initial immune response elicited by TNFα and more heavily focused on innate immune activation via toll-like receptors (TLRs) and TLR signalling. In several studies, progression of sepsis required the TLR adaptor molecule MyD88 and antibody mediated inhibition of TLR4/MD2 prevented lethality induced by CASP (31;32). IL-12 and inducible nitric oxide synthase (iNOS) offer some antibacterial and immunoprotective effects since mice genetically deficient of IL-12 and iNOS are more susceptible to CASP-induced sepsis (33). Additionally, exogenous addition of complement factor C3 (34) and activated protein C (35) offered cytoprotective and anti-inflammatory effects against CASP-induced lethality.

The CASP model of polymicrobial sepsis and peritonitis appears to have substantial utility, however our understanding of signalling pathways and the pathobiology which results in disease in this model are lacking. Animals subjected to CASP appear to mount a rapid, stronger immune response than animals subjected to CLP but further experimentation is required to determine the efficacy of using CASP as a sepsis model. Undoubtedly, the use of CASP will increase in prevalence as the pathophysiology and underlying mechanisms of disease which produce the septic conditions are uncovered.

3.3. CLP vs. CASP

Notable differences in the host response to CLP versus CASP-induced sepsis have been documented. The progression of sepsis in the CLP model involves TNFα-mediated activation of the immune response while the CASP model is less dependent on the initial immune response elicited by TNFα and more so dependent on TLR activation and signalling (12;25;29). One study comparing the two distinct models found that bacterial counts of peritoneal lavage, liver, lung, and serum levels of TNFα, IL-1β, and IL-10 increase steadily over a 24 hour period in the CASP model and were significantly higher than that of mice subjected to CLP (24). In this study, the authors observed continuously low bacterial counts and cytokine levels at all time-points as well as abscess formation around the cecum in mice subjected CLP. In light of these observations, it has been suggested that CASP is a true model of peritonitis with early SIRS, while CLP more closely mimics abscess formation (24;25). Alternatively, others interpret the rapid elevations in systemic cytokines, bacterial counts and strong immune response following CASP to be comparable to that observed in endotoxemia models and the inflammatory reactions characterized by protracted cytokine profiles following CLP to more closely reflect clinical sepsis (12).

Discrepancies between the host response to CLP versus CASP have been observed in studies using genetically-modified animals, although consensus on the interpretation of these results is lacking. While TNFα-deficient mice were protected in a CLP model, TNFRp55/TNFR1-deficient mice did not appear to have an altered resistance to CASP-induced sepsis (26;36). However, the differences observed may be due to the incomplete abolishment of TNFα activity as these mice may be deficient in only one of two TNF receptors, abolishing the cytotoxic TNFR1 and leaving the protective TNFR2 intact (37). Additionally, IFNγ exhibited protective effects in a CASP model and not in CLP-induced sepsis (26;36). Abolishing cytokine IL12p40 rendered the host more susceptible to CASP-induced sepsis while the same deficiency was found to either have no significant effect in some CLP studies (36) or increase susceptibility to sepsis in others CLP studies (38). The discrepancies in experimental outcomes of studies using CLP and CASP may be results of differences in the host response between these sepsis models, and an incomplete understanding of the pathophysiology of each model. Careful consideration should be taken to choose an appropriate model to address the primary research question to be investigated.

3.4. Limitations of current models

Earlier models of endotoxemia and bacterial inoculum fall short in modelling the complex changes which occur in clinical sepsis. Many cardiovascular, respiratory, metabolic, hormonal, inflammatory, innate and adaptive immune changes associated with the spectrum of septic conditions cannot be sufficiently reproduced by a single injection of endotoxin or bacteria (12). Injection of isolated microorganisms fails to mimic the host response to the diversity of causative agents in clinical sepsis. A specific instance is the injection of an endotoxin like lipopolysaccharide (LPS, a component of the cell wall of gram-

negative bacteria) (4). LPS endotoxemia is dependent on TLR4 signalling and represents one specific aspect of the immune response, not the complex interactions of multiple signalling pathways during the progression of sepsis. Endotoxin injection and bacterial inoculum are followed by rapid elevations in cytokines which are much higher than what is observed in human sepsis (4). These models are more reflective of endotoxic shock rather than sepsis due to the overload of endotoxins in murine animals which exhibit a much higher endotoxin resistance than humans, further decreasing the clinical relevance of these studies (12). Moreover, these increases are transient, occurring in a short time span, and fail to reflect the complex physiological response in clinical sepsis.

Although surgical polymicrobial sepsis models show a greater clinical relevance than earlier models of endotoxemia and bacterial or fecal injections, both CLP and CASP are not without limitations. While the severity of experimental sepsis can be controlled by modifying certain elements of a CLP protocol (e.g. length of ligated cecum) these factors may also decrease the consistency between animals and reproducibility of the study, as the amount of cecum ligated may vary between subjects (3). More contributing factors to inter-animal variability include differences in the amount of fecal content in the cecum at the time of surgery, the size of cecum of each animal, and bacterial flora in different animals. Moreover, conflicting findings over the effect of the number of punctures on disease severity further highlight differences that may result due to surgical manipulation at the hands of different experiments (24). Another limitation of CLP and CASP……..

Another limitation of CLP and CASP is the inter-study variations due to differences in protocols used between investigators. The number of cecal punctures and sizes of needles used to perforate the bowels vary between studies using the CLP model. Likewise, differences in the diameter of catheter used, location of stent insertion and suturing in the CASP model also influence disease severity (24;25). While some of these limitations will inevitably affect the consistency and reproducibility of sepsis studies in animals, standard protocols can be enforced to reduce potential discrepancies.

4. Sepsis in large animal models

Small size, shorter reproductive cycles as well as less housing and maintenance costs are some advantages of utilizing small animals for scientific research. However certain physiological features of small animals vary considerably from its human counterparts (39). In addition, serial tissue and blood samples cannot be extracted from small animals, increasing the number of animals required to study whether an affect exists. Large animals, on the other hand not only allow serial sampling but also have very similar immunological and physiological functions to humans, rendering them better subjects to model clinical sepsis and drug testing. The Food and Drug Association (FDA) also recognizes the value of large animals requiring all new drug applications to include data from at least one non-rodent animal. In this section the rabbit, canine, porcine, ovine and non human primate models of sepsis and their limitations will be discussed.

4.1. Rabbit models of sepsis

A rabbit model of pneumococcal sepsis was developed in 1970. It encompasses several aspects of clinical sepsis including increased cardiac output and body temperature (40;41). This model involves the use of *Diplococcus pneumoniae* to induce sepsis and an inoculum of 1 ml of medium containing rabbit blood and tryptase soy broth with 10^8 to10^9 colony forming units is administered intraperitoneally (40) Several other rabbit models of sepsis were developed to study lung injury. Matute Bello et al. observed that a dose of 10^9 cfu/clot induced a more persistent infection compared to 10^8 cfu/clot, while inoculation of 10^{10} cfu/clot had lethal effects in a rabbit model of peritonitis (42). In this model, polymorphonuclear leukocyte (PMNs) function in the peritoneal cavity was perturbed suggesting potential contribution in the development of septic shock (42). Overall, the rabbit model of sepsis has been used to investigate physiological and immunological responses during sepsis and septic shock (43).

4.2. Canine models of sepsis

The canine models of endotoxemia and bacteremia have been used extensively to study cardiovascular function during sepsis. Dogs subjected to septic shock, show responses that parallel human sepsis. For example, there is a severe but reversible decrease in systolic ventricular function (44), a 32-108% increase in cardiac output, decrease in mean arterial pressure, and leukocytosis as well as increase in plasma epinephrine and norepinephrine levels during septic shock (45). Cytokine profiles in the canine model of endotoxemia also mirror those reported in human sepsis patients with a 62 fold increase in IL-6 mRNA in peripheral blood mononuclear cells (PBMC) and 4.5 fold increase in TNFα within the first hours compared to controls (46).

Despite the analogous physiological responses, there are several limitations of this model pertaining to clinical relevance. Canines have an adrenergic sensitive sphincter around the hepatic vein which constricts during sepsis increasing intestinal venous pressure and damaging the mucosal barrier, increasing its relevance to a gut injury model (47;48). Another important limitation is the resistance of canines to endotoxins and the requirement of high LPS dosage to induce sepsis (39;49). This requirement for increased endotoxemia results in a severe hypodynamic response in canines (50) which does not mimic hyperdynamic human sepsis.

4.3. Porcine models of sepsis

Pigs are popular animals for since they are readily available and relatively easy to handle. Continuous infusion of live bacteria, endotoxins as well as CLP are some of the methods used to induce sepsis in pigs. Porcine models of sepsis have been used extensively to investigate therapeutic agents focusing on improving renal, hepatic, intestinal and cardiovascular function (51-53). For instance, using a porcine model of LPS induced shock, Cohen et al. observed that increasing levels of nitric oxide (NO) improves renal blood flow

and glomerular filtration rate (51). In addition, the porcine model of fecal peritonitis has been previously used to investigate the impact of adding low dose arginine vasopressin (AVP) to norepinephrine infusion for improving organ function (52). In the AVP treatment group, renal function was significantly improved, and significantly less hepatic apoptosis compared to the group treated with norepinephrine alone, suggesting that the addition of AVP to norepinephrine improves renal function (52).

The porcine model of fecal peritonitis has also been used to study acute lung injury associated with sepsis. Septic pigs have decreased left ventricular function, respiratory dysfunction as well as hemorrhage, pulmonary congestion associated with neutrophil infiltration characteristic of acute lung injury (53). This model has been further used to study effects of certain therapeutic agents such as N-acetylamrinone and L-arginine (54;55).

Due to the close similarity between pig and human anatomies, these animals have also been utilized to develop techniques commonly performed on sepsis patients such as laparoscopy (56). Porcine models of neonatal sepsis also closely mimic the clinical course of neonatal sepsis with a significant decrease in systemic blood pressure (71 ± 3 mmHg in sepsis and 64 ± 3 mmHg in control at 3 h) and increases in serum levels of endotoxins, TNFα, and IL-6 (57). Anatomical and physiological similarities between porcine and human anatomy have allowed for the successful testing of techniques and study of therapeutic agents which translate to human sepsis.

4.4. Ovine models of sepsis

In sheep, infusion of endotoxins (58), live bacteria as well as administration of fecal content into the abdominal cavity are some methods used to induce sepsis. The ovine model of sepsis has distinct similarities to human sepsis and several advantages over other animal models specifically regarding cardiopulmonary responses. One similarity between sheep and human sepsis is the biphasic cardiovascular profile. In endotoxemia models of ovine sepsis, two phases of cardiopulmonary function are observed (59). Within the first hour, the animal is in a hypodynamic state with a low cardiac index, myocardial contractility, high mean pulmonary arterial pressure, and pulmonary vascular resistance (59). The first phase is followed by a hyperdynamic state with significant increase in cardiac output (59).

The ovine is also a popular specie to study sepsis associated with lung injury. Daniel Traber is a pioneer in developing the ovine model of 'smoke injury' which involves insufflating sheep with smoke from burning cotton cloth (60) and the 'ovine burn model' which involves third degree burns and smoke injury (61). Smoke inhalation is induced using a modified bee smoker filled with 40 g of burning cotton cloth attached to a tracheostomy tube through a modified endotracheal tube (61). Based on similar principles, the ovine model of 'smoke inhalation and septic shock' was developed to study sepsis associated with pneumonia that develops due to acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) after smoke inhalation (62). In this model, the aforementioned technique of 'smoke inhalation' is used to induce lung injury (63), followed by instilling bacteria such as *Pseudomonas aeruginosa* into the lungs (63;64).

Using the 'ovine smoke inhalation and septic shock' model several therapeutic agents have been investigated for sepsis management. Necrosis and apoptosis in this model were improved by administration of WW-85, a peroxynitrite decomposition catalyst. Other notable changes include improved gas exchange, decreased levels of myeloperoxidase (MPO) and lung 3-nitrotyrosine (65). There was also an overall improvement in pulmonary function suggesting that blocking nitric oxide-peroxynitrite pathway may ameliorate some effects of septic shock (65).The ovine model of sepsis and septic shock has made important contributions in the areas of cardiovascular (66) and pulmonary research as well as in determining the efficacy of fluids (58) and therapeutic agents (67), such as anticoagulants (68) and recombinant human protein C (69) to improve sepsis outcomes.

4.5. Non-human primate models of sepsis

Due to genetic similarities to humans, non-human primates are an ideal model for testing species-specific therapies (70). The method of inducing sepsis and septic shock in baboons utilizes an intravenous infusion of live *E.coli* (70). Using the baboon model of sepsis, several therapeutic discoveries have been made, the most prominent one being Activated Protein C (APC) (71;72). Taylor et al. reported that co-administration of APC with E. coli at lethal doses reduces coagulopathic and hepatotoxic effects induced by E. coli. This blocking of lethal effects of *E.coli* disappeared when protein C activation was blocked *in vivo* (73). More recently Xu et al. also reported that histone levels increase following *E.coli* administration in baboons. Extracellular histones released in response to inflammatory stimuli and during sepsis contributes to endothelial dysfunction, organ failure and death (71). Xu et al. discovered that baboons co-administered with both APC and *E.coli*, were rescued from mortality (71). The success of APC in baboon model of sepsis, allowed for its use in the clinical setting, until its recent withdrawal from the market following a further phase III trial. Despite the advantages associated with baboon models of sepsis, risk of infectious disease transmission, high housing and maintenance costs, as well as the ethical concerns deter many investigators from utilizing these animals for sepsis research (49).

4.6. Limitations of large animal models of sepsis

Large animal models have provided tremendous insight into the pathophysiology of sepsis, however there are certain limitations to the use of these animals. Due to their large size, they are also more difficult to handle, house, and anaesthetize and in the case of primates, pose risks of cross transmissible diseases (47).

5. Co-morbidities and sepsis

There have been several therapeutic agents with positive outcomes in animal models however majority failed to show efficacy in clinical settings. This disconnect is partially due to discrepancies in translating findings from animal models to clinical sepsis. Defined human target populations and established severities of sepsis would allow for more realistic and applicable animal modelling.

Clinical studies report that individuals with co-morbidities such as diabetes are at a higher risk of mortality and morbidity from infectious diseases (74-77). It has also been estimated that greater than 50% of septic patients have at least one additional co-morbid condition (78). Therefore, incorporating co-morbid conditions into animal models is one method by which modeling clinical sepsis in animal models could be improved.

5.1. Sepsis and diabetes

Several animal studies and clinical trials have demonstrated that diabetes increases the risk of infections and mortality (74-77). A retrospective cohort study from Ontario, Canada by Shah et al. reported that almost half of the diabetic patients in their study (n=513,749) had a minimum of one case of infectious disease for which they were either hospitalized or received a physician claim (74). It was also found that bacterial infections and infections in general were also more commonly found in diabetics (74). From animal models of type II diabetes it is known that sepsis induced inflammation is more severe in diabetic animals (79). Septic diabetic animals also have increased bacteria load (80;81), altered levels of cytokine expression (81;82) and dysfunctional immune cells such as PMNs contributing to poor outcomes.

Studies in animal models of obesity parallel the findings in diabetic animals. Strandberg et al. reported that mice fed a high fat diet (HFD) with saturated fats, had higher mortality rates when challenged with *Staphylococcus aureus* compared to mice fed a low fat diet (LFD) (83). The HFD fed group had increased bacterial load, decreased immune cell-mediated clearance of bacteria, and significantly elevated pro and anti-inflammatory cytokine levels compared to mice on a LFD (83). Therefore obesity can influence the response of immune cells to infections such as sepsis and thus have direct impact on sepsis outcomes.

5.2. Urinary tract infection and sepsis

The urogenital tract is the focus of infection in approximately 25% of all sepsis cases (84). Urinary tract infection (UTI) associated sepsis is predominantly found in elderly individuals, diabetics and immuno-suppressed patients (85). Several animal models have been developed to study different aspects of these co-morbid conditions. Harberg et al. developed a model of *E.coli* induced UTI by infecting urinary bladders of female mice by administrating bacteria via a urethral catheter (86). Pyelonephritis isolates (HU734) and normal fecal (414) *E. coli* were used to induce infection, where the pyelonephritis strains were discovered to remain in the system longer (86). UTI associated sepsis animal models have also been used to test potential therapeutic strategies. Reid et al. investigated whether competitive exclusion of uropathogenic bacteria would occur when animals were treated with indigenous bacteria (87). In female rats with chronic UTI induced by periurethral injections of agar beads with bacteria, rats exposed to indigenous bacteria , *Lactobacillus casei*, 21 days before the uropathogen challenge, had no pathogenic bacteria or immune responses in the bladder and kidney for up to 60 days (87). Given the prevalence of UTi-associated sepsis in the clinical setting, using animal models which involve co-morbidities and common clinical infections will increase the relevance of these animal studies.

5.3. Genetics and sepsis

Several studies have identified the implications of genetic variance, for immune responses to sepsis. Even while comparing across different strains of septic mice, mortality rates, liver MPO activity, metallothione mRNA, leptin as well as IL-10 levels are significantly higher in C57BL/6J compared to A/J mice (88). Thus, genetic differences in mice and possibly humans are associated with differences in the inflammatory processes initiated in response to infection that ultimately affects sepsis outcomes (88).

Transgenic animal models have also been utilized extensively in sepsis research (89). Transgenic apolipoprotein (ApoE) polymorphs, ApoE3TR and ApoE4TR, generated by replacing mouse ApoE allele with its human counterpart coding sequences were used to investigate if ApoE genotype affects sepsis outcomes (89). ApoE is a ligand for low density lipoprotein (LDL) receptor, responsible for clearance of VLDLs and chylomicrons residues. ApoE plays key role in lipid metabolism (90) and mediates removal of inflammatory apoptotic substances (91). Mice with the human APOE4 allele have greater inflammatory response (with two to four fold increase in synthesis of cytokine) as well increased time to mortality (89).

ApoE knockout mice (apoE -/-) generated by gene targeting are more susceptible to endotoxemia and K. *pneumoniae* compared to LDLr-/- mice implicating the role of apoE in modulating LPS induced inflammatory responses (92). Haraguchi et al. have reported using the ApoE-/- strain that administration of pioglitazone suppresses inflammation and improves survival in these mice, providing support for the use of peroxisome proliferator-activated receptor gamma (PPAR-γ) agonist as potential treatment option for severe sepsis (93).

Other strains of transgenic mice have also been utilized for sepsis research such as mice with inactivated beta$_2$ integrin CD11b. These transgenic mice are partially protected against micro-vessel permeability and edema formation, suggesting key role of Cd11b in lung PMN sequestration and vascular injury during the early phase of gram-negative sepsis (94). Lectin-like oxidized low density lipoprotein receptor 1 knockout (LOX-1-/-) mice have been used to investigate the role of LOX-1 in sepsis induced mortality. Wu et al. reported that LOX-1-/- mice with CLP-induced sepsis had decreased systemic inflammation, neutrophil migration to sites of infection, levels of pro-inflammatory cytokines, and lung edema as well as increased bacterial clearance implicating the key role of LOX-1 in systemic inflammation (95). Important findings have also been discovered in toll like receptor knockout mice (TLR-/-) . Toll like receptors (TLRs) recognize different components of bacteria cell wall and mediate immune responses to infection. TLR-4 for instance interacts with LPS, activating the expression of target genes. TLR-/- knockout mice have been utilized to study dendrititc cell maturation and cytokine production (96), infections of the urogenital organs (97) and defense against infections such as murine tuberculosis (98) among several others aspects of adaptive and immune responses.

Transgenic mice such as ob/ob have also been used to examine the effects of leptin deficiency on sepsis outcomes (99). Leptin deficiency, in ob/ob mice, is associated with

greater organ dysfunction and increased mortality rates during sepsis (99). Leptin signaling appears to improve survival and may be required for immune responses, implicating therapeutic potential for leptin analogues for sepsis. Thus transgenic mice have made important contributions to Improve our understanding of sepsis at physiological, immunological and cellular levels.

6. Emerging preclinical research in sepsis: Neutrophil Extracellular Trap (NET) formation

An emerging area of sepsis research involves the formation of neutrophil extracellular traps. In 2004, Brinkmann et al. characterized a novel mechanism of innate immunity exhibited by neutrophils. Upon stimulation by endotoxins or pro-inflammatory mediators, activated neutrophils released chromatin material composed of a DNA backbone and antimicrobial granular proteins in the form of neutrophil extracellular traps (NETs) (100). NETs ensnare circulating microorganisms, preventing further dissemination of pathogens in the vasculature while providing a scaffold for neutrophil granular proteins. This creates a high, local concentration of proteins with antimicrobial properties (100) and allows for microbicidal synergy, concentrating their ability to disarm and kill microorganisms (101).

6.1. NETosis

The formation of NETs, recently coined NETosis (100;102) is an active process. Cleavage of histones by neutrophil elastase is sufficient to cause decondensation of nuclear material (103) which can be observed before disintegration of nuclear and granule membranes. Chromatin material mixes with nuclear and granular proteins with potent antimicrobial properties and is released extracellularly (102;104) forming stretches of DNA and globular proteins with diameters of 15-17 nm and 25 nm respectively which can aggregate to form thicker threads around 50 nm in diameter (100). The process of NETosis is distinct from that of necrosis (the plasma membrane remains intact while nuclear and cytoplasmic granular components mix) and that of apoptosis (no phosphatidylserine exposure signaling phagocytosis, or nucleosomal cleavage) (102;105).

NETosis can be experimentally induced by exposure to endotoxins (e.g. LPS), gram-negative and gram-positive bacteria, fungi, pro-inflammatory mediators (e.g. IL-8, phorbol myristate acetate or PMA a protein kinase activator), and activated platelets (103;106-111). NET formation in experimental conditions results in the release of proteins which degrade virulence factors expressed on the pathogen surface and create a toxic environment for invading microorganisms (102;107;112-115). NET-associated azurophilic granule proteins which have antimicrobial or immunomodulating effects include histones with specific post-translational modifications (and histone cleavage products like buforin), cathepsin G, elastase, MPO, pentraxin, gelatinase (matrix metalloproteinase-9), catalase, lactoferrin, peptidoglycan recognition proteins (PGRPs), and bactericidal permeability-increasing protein (BPI) (100;107;116). NETs have also been shown to kill infectious organisms and

impair pathogenic invasion of gram-negative bacteria (e.g. *Shigella flexneri), gram-positive bacteria (e.g. Staphylococcus aureus, Group A streptococcus),* fungi (e.g. *Candida albicans)*, and even viruses (e.g. influenza A virus) (100;102;106;107;107-110;112;114-117).

6.2. NETosis in infection and sepsis

In 2007, a landmark paper published by the Kubes group characterized the mechanism by which platelet-neutrophil interactions contribute to pathogenesis of severe sepsis. It was observed that LPS-induced endotoxemia in mice as well as plasma from severe sepsis patients were able to trigger NET formation but at a cost to the host. Upon detection of TLR4 ligands (e.g. LPS), platelets bind to neutrophils adhered to the endothelium (118). Within minutes, TLR4-dependent platelet-neutrophil interactions resulted in the robust release of granules and DNA in the form of NETs the integrity of which was maintained under flow conditions reflective of physiologic shear forces in the microvasculature (0.5 dyne/cm^2). NET-mediated bacterial clearance through trapping and ensnaring of bacteria was primarily observed in liver sinusoids and lung capillaries, areas with the greatest capacity for immobilizing bacteria due to the decreased lumen size of vessels (118). However, formation of NETs is not without a cost as it was also found to cause cellular and tissue damage to the host. *In vitro*, NET formation resulted in endothelial cell damage marked by increased staining of propidium iodide, a nucleic acid stain impermeable to viable cells (118). *In vivo*, NET formation was also found to cause hepatotoxicity in LPS-challenged mice indicated by the decreased perfusion of liver sinusoids and increase in levels of alanine aminotransferase, associated with platelet-mediated neutrophil activation. Given these findings, the authors propose that platelets act as a sensor or barometer (partially via TLR4 receptors) for pathogens in the blood, inducing a last resort immune response mediated by neutrophils to ensnare and kill bacteria at the expense of the host's tissues (118). In the already immune-deregulated state of the septic host, NETs can exacerbate sepsis by releasing high concentrations of potent proteases, inducing further endothelial damage, and forming a chromatin meshwork, trapping host cells (erythrocytes, leukocytes, and platelets) which can potentiate inflammation, coagulation, ischemia, and hypoxia in downstream tissues.

6.3. NET pathogenicity

NETs induce pathogenic effects via multiple mechanisms. Platelet-mediated NET formation trapped platelets, leukocytes, and red blood cells causing microvascular plugging and areas of ischemia in downstream tissues (118;119). Ischemic conditions increase the production of IL-8 (120) and reactive oxygen species (121) both of which can induce further NET formation (122). Several lines of evidence also suggest that NETs exert pro-coagulant effects in sepsis and other conditions of deregulated coagulation and inflammation. In studies of thrombosis, NETs were found to interact closely with fibrin and platelets via von Willebrand factor, fibronectin, and fibrinogen, effectively stabilizing platelet-rich clots (119;123). NETs perfused with blood or platelet-rich plasma stimulated platelet aggregation and promoted thrombus formation (124). Another *in vivo* study observed that both DNA and RNA provide a surface template on which activation of the contact pathway via XIIa/XIa promoting fibrin-

rich thrombus formation and decreased plasma clotting times (125). Antimicrobial proteins associated with NETs also exert pro-coagulant effects which are pathogenic to the host. Positively-charged histones promote red blood cell accumulation and platelet aggregation by electrostatic interactions with the negatively-charged cells (126). NET-associated histones also impair the natural anticoagulant protein C, activate platelets, and induce thrombin generation via platelet TLR2 and TLR4-mediated mechanisms (124;127-129). Additionally, histones induce prothrombinase activity, increased P-selectin expression, phosphatidylserine exposure, and FV activation (127).

NETs and NET-associated proteins have also been found to exert pro-inflammatory effects. MPO released during NET formation binds the negatively-charged, proteoglycan-rich endothelium, inducing endothelial cell damage and vascular permeability (130;131). Incubation of NETs with human platelets and THP-1 cells resulted in the release of pro-inflammatory cytokines including IL-1β, IL-8, and TNFα (132).

The pathogenic nature of NETs is also supported by studies showing improved outcome associated with NET destruction via DNase administration (133). Furthermore, DNaseI-deficient mice develop lupus-like symptoms induced by NET formation, indicating that NET removal is likely a crucial process in the proper immune response (123). The formation of NETs and release of NET-associated proteins (e.g. histones) potentiate the pro-inflammatory and pro-coagulant response, both of which contribute to end organ morphology in sepsis. These changes include neutrophil adherence to microvascular endothelium, inflammatory infiltration, vacuolization of epithelial and endothelial cells, fibrin deposition, microvascular ruptures with intra-alveolar hemorrhaging, and the formation of platelet-rich micro- and macrovascular thrombi (118;127;129;134;135). While clinical isolates have been shown to have the ability to generate NETs *in vitro,* there is to date no clear evidence to show whether this phenomena occurs in septic patients, or whether this is associated with organ dysfunction or mortality.

7. Translation of preclinical studies to clinical outcomes

Despite the success of many therapeutic agents in improving outcome in preclinical studies of sepsis, many have failed to demonstrate efficacy in the clinical setting. To illustrate, studies investigating the efficacy of anti-TNF therapies (5;5;136;137) and activated protein C (APC) were promising in animal studies, but this success failed to translate to clinical outcomes (35;73;138). Neutralization of TNF was beneficial in some models of endotoxemia challenge with viable gram-negative bacteria (e.g. group B streptococci) (139-145) but in other preclinical studies where microorganisms such as Candida, S. pneumoniae, Listeria, or mycobacteria were used, neutralizing TNF exacerbated outcome in animals subjected to the pathogenic challenge (36;70;136;137;146-149). In complex models of sepsis, no consistent harm or benefit was validated and some clinical studies found an increased mortality associated with anti-TNF therapy (150;151). Similar to anti-TNF therapy, some preclinical studies demonstrated the protective or therapeutic effect of APC (35;73;138) but these results failed to translate in some clinical studies (72). The therapeutic efficacy of anti-TNF therapy

and APC in preclinical studies is largely influenced by the animal model used. Thus the appropriate translation of findings and inferences from preclinical studies to clinical outcomes in sepsis remains a heavily debated area.

7.1. Critical appraisal of preclinical sepsis studies

Limitations in both the use of animal models and design of experimental studies contribute to the poor translation of preclinical animal studies to clinical sepsis. Some limitations are inherent to the use of animals to model any clinical condition. It is commonly the case that young animals of a specific gender, species, genetic background, and nutritional status housed in a pathogen-free, sterile facility unexposed to the natural environment are used (5). Many of these elements are tightly controlled to maintain consistency at the expense of clinical relevance. However, an attempt at balancing both consistency and clinical relevance can be made if the investigator designs treatment groups for different animals (e.g. separate treatment groups for females and males, groups for young and aged mice, etc.) given the heterogeneity of the sepsis patient population.

Other limitations may be appropriately addressed by establishing clear research questions and implementing an experimental protocol which would adequately investigate these questions. For instance, if the objective of an animal study is to test the clinical applicability of a therapeutic agent, it would be more clinically relevant if therapies currently used for the management of sepsis including the adequate administration of resuscitation fluids, antibiotics, and supportive therapies (14) were incorporated into the experimental protocol of the animal study. Additionally, the experimental protocol of animal studies can be modified to include clinically relevant management procedures such as constant monitoring and assessment for hemodynamic parameters, tissue perfusion, or dehydration as would occur in clinical sepsis. Other factors which may contribute to the gap between findings in experimental and clinical studies include the time at which therapeutic agents are administered, the lack of staging of sepsis to reflect disease progression at different severities on the sepsis continuum or different patient populations, and the risk of experimenter bias in animal studies (5;12)

7.2. Limitations to the clinical relevance of animal studies

7.2.1. Age

It is commonly the case that murine studies of sepsis utilize 8 week-old mice, the physiological equivalent of a young adulthood in humans. However, the sepsis patient population consists largely of patients over 60 years of age which is not adequately represented in sepsis literature using animal models (152). There are limitations to examining the true pathophysiology and clinical treatment of a condition which occurs most commonly in the aging population when findings are extrapolated from preclinical studies using young, healthy mice exclusively. For example, aging is associated with increased apoptosis of rapidly dividing epithelial cells of the gut and spleen in animal sepsis (153).

This may contribute to the increased mortality in aged septic mice, a factor which would not accounted for when using young animals to model sepsis occurring in an aging population. The effect of age in sepsis in a clinical setting can be appreciated in the PROWESS trials. Substantial difference in absolute risk reduction of mortality were found to be associated with age in clinical sepsis (154). Age should be considered in animal studies of sepsis to increase the clinical relevance to human sepsis.

7.2.2. Gender

In addition to limitations produced by using young animals exclusively, male animals are almost exclusively chosen in intra-abdominal sepsis studies which poorly reflects the incidence of clinical sepsis in both genders around 40% of which is female (155). Male mice are often chosen over females to avoid confounding effects and variables posed by varied expression of biomarkers and circulating cells associated with different phases of the estrous cycle. For instance, steroid hormones are implicated in the expression of adhesion molecules resulting in different peripheral blood leukocyte concentrations and an altered coagulant response (156;157). Neutrophil concentrations and response to stimulation also vary considerably during different phases of the estrous cycle (158). For consistency, male mice are often chosen of sepsis studies, despite the similar occurrence of sepsis in males and females however, an investigator should consider, if feasible, treatments with one group for each gender of animal used.

7.2.3. Fluid resuscitation

The importance of early goal-directed treatment including adequate fluid resuscitation and treatment with antibiotics has been thoroughly demonstrated in severe sepsis and septic shock (159). Correction of hemodynamic abnormalities and hypovolemia associated with sepsis is integral to reducing mortality rates and improving sepsis outcomes. Hypovolemia compromises tissue oxygenation due to inadequate blood flow within the microvasculature and is the prime cause of organ dysfunction and failure (159). In order to differentiate pathology due to sepsis from pathology resulting solely from circulatory decline and lack of hemodynamic support, the administration of balanced fluids is crucial. This is supported by findings showing significant differences between the hemodynamic profiles of under-resuscitated animals versus those with adequate supportive fluids, and aggressive fluid resuscitation was required to replicate hemodynamic profiles observed in patients with severe sepsis (47). In canines with septic shock, animals that received combination therapy of antibiotics and cardiovascular support (via fluid resuscitation and dopamine) had a 43% improvement in survival rates compared to septic animals treated with either therapy alone (160;160). Furthermore, experiments elucidating the effects of various fluid regimes on resuscitation in sepsis have demonstrated that lactated Ringer's crystalloid solution but not saline-based solutions reduced sepsis-induced leukocyte recruitment in the liver of mice subjected to CLP (161). Based on these studies, an appropriate fluid regime which would account for surgical losses and provide adequate hemodynamic support to maintain circulatory and cardiovascular function, as would occur in the management of clinical sepsis, should be considered in preclinical studies of sepsis (12).

7.2.4. Patient heterogeneity

The human septic population is diverse and highly heterogeneous. Clinical cases of sepsis are often much more complex than sepsis induced in animal studies. The diversity of infectious agents as well as sites of infection in clinical sepsis are not always reflected in animal studies, factors which should be considered in the translation of preclinical to clinical studies. For instance, clinical sepsis may result from trauma and subsequent fecal spillage into a sterile peritoneum or staphylococcal bacteremia in an elderly patient with congestive heart failure. Evidently, animal models like those inducing sepsis via injections of endotoxic bacterial components or even live bacteria fail to induce the range of conditions observed in a septic patient (5). To decrease this gap, one may choose to consider introducing cormorbidities as previously described or other trauma or infectious injuries to animal sepsis models.

Animal	Species, genetic background, gender, age, nutritional status following insult, comorbidities incorporated in model
Source of Infection	Single versus multiple organisms, local versus systemic challenge, addition of adjuvants, presence and extent of tissue damage from challenge
Intervention	Dose and timing of intervention compared to septic insult (administered before, concurrent with, or following insult)
Co-interventions	Fluid resuscitation, antibiotics, analgesics, source removal
Experimental Design	Risk bias (blinding or randomization methods are used), assay methodology
Markers of Outcome	Parameters used as markers of outcome (choice of biomarker and quantification methods, physiological response, inflammatory parameters, survival)

Table 1. Limitations to the Clinical Relevance of Preclinical Studies

7.3. Summary of limitations

Factors which limit the translation of preclinical studies to clinical outcomes include the following (5): the animal used for experimental studies (species, genetic background, gender, age, nutritional status following insult, comorbidities incorporated in model), the source of infection (organism, local vs. systemic challenge, adjuvants, tissue damage from challenge), dose and timing of intervention (administered before, concurrent with, or following insult), co-interventions (fluid resuscitation, antibiotics, analgesics, source removal), experimental design (risk bias, blinding, randomization, assay methodology), and parameters used as markers of outcome (quantification and choice of biomarker,

physiological response, inflammatory parameters, survival) which can significantly alter response to treatment. These potential limitations are summarized in Table 1 adapted from Marshall et al, 2005.

7.4. Considerations to improve the translation of preclinical studies to clinical outcomes

It is evident that experimental design greatly influences the findings of both experimental and clinical studies in sepsis. One method to increase the transparency, reproducibility, consistency, and efficacy of sepsis research is to increase standards for reporting of animal sepsis studies. The Consolidated Standards of Reporting Trials (CONSORT 1996, 2001, and 2010) statement is a rigorous and highly standardized approach to conducting clinical research which addresses variability of results due to the study design and reporting of clinical trials (162-164). In an attempt to limit the variability in both the use of animal studies and reporting of results, Marshall et al. propose that a similar checklist should be required for preclinical studies (5). The checklist includes the following variables which should be explicitly described and recorded in detailed manner to ease pooling of results from various preclinical studies, define a framework from which to understand divergent results, improve the consistency of reporting of results, and enhance the reliability of reported results. The standards for reporting animal research in bioscience were raised further in 2010 with the publication of the ARRIVE guidelines, Animals in Research: Reporting *In Vivo* Experiments (165). Below is an adaptation of the Consolidated Standards of Reporting Trials Checklist for Preclinical Studies proposed by Marshall et al. in 2005 with descriptions of methods by which these reporting standards could be maintained as suggested by Kilkenny et al. (Table 2).

7.4.1. Sepsis definition

In the design and reporting of a preclinical sepsis study, a basic but crucial aspect that one should consider is the very definition of sepsis, the conditions and presentation of which differ from one animal model to another. Clinical sepsis is defined by meeting specific criteria which involve inflammatory, hemodynamic, organ dysfunction, and tissue perfusion variables. Sepsis is no longer defined solely by changes in physiological parameters (e.g. body temperature, heart rate, respiratory rate, and white blood cell count) which are common to many other conditions. Likewise, defining sepsis based on levels of several biomarkers of inflammation, for instance fails to capture the complexity of cardiovascular, hormonal, metabolic, innate and adaptive immune changes which occur in this heterogeneous condition. Although it would be helpful to document changes in biomarkers and physiological parameters (e.g. heart rate, etc.), current preclinical studies are limited as the criteria which define sepsis (and the severity) in various animals have not been clearly elucidated. There is currently no consensus over the physiological parameters, levels of biomarkers, or variables of end organ health which would indicate sepsis in an animal although these parameters are much more clearly outlined by clinical

sepsis definition guidelines (1). This limitation will need to be addressed in future preclinical sepsis studies. Other parameters which may be informative of the septic condition in animals include appearance (perfusion of mucous membranes), urine production, and bacterial cultures from the blood, peritoneal fluid, and local site of infection, which may be considered in the design of animal studies. Successful animal studies should establish a clear definition of the conditions which would indicate a septic animal, explicitly defining the severity of disease induced as part of the study protocol (167).

Sepsis Definition	Clear sepsis definition as indicators of sepsis, severe sepsis, or septic shock in the animal used (166)
Animal	Species, strain, genetic background, gender, age, weight, handling, housing, feeding conditions
Experimental Design	Method of sepsis induction (details which indicate severity), intervention (timing and dose), experimental methodology, controls included, randomization methods, blinding of experimenter
Analytic Plan	Primary and secondary endpoints, power calculations for sample size determination, intention-to-treat analysis, criteria for animals excluded from studies (substitute endpoints)
Co-Interventions	Resuscitation fluids, antibiotics, source removal, feeding
Results	Flowchart of included and excluded animals, establish mortality rates in studies conducted

Table 2. Checklist for the Reporting of Animal Studies

7.4.2. Experimental design

In the interests of reproducibility and reliability, the method of sepsis induction, intervention (timing and dose), experimental protocol, inclusion of proper controls, randomization methods, and methods of experimenter blinding should be documented in detail. Although experimental methodologies are commonly recorded, there is a significant lack of attention to reduce experimenter bias by randomization and blinding, despite the confounding effects recently addressed in a systematic review of literature of studies using animal sepsis models. It was observed that only 2% of systematic reviews and meta-analyses appraised the risk of bias or clinical relevance of the underlying animal

research despite a significant proportion of the literature extrapolating results from animal studies to clinical sepsis (168). In one review of studies using animal models, more than 80% of research papers surveyed did not report the use of methods to minimize the risk of bias by the investigator (165;169). To improve the translation of inferences and findings from experimental sepsis to clinical sepsis in future studies, one should consider reducing the effects of experimenter bias by randomizing animals in the treatment groups of the study and blinding the experimenter to the intervention provided given that implementation of these elements would not compromise the protocol or results of the study (5;166;167).

7.4.3. Randomization

In the absence of randomization, the results of clinical and animal studies may be unintentionally biased by factors which may influence study outcomes. Nonrandomized clinical studies have frequently shown a larger treatment effect than randomized control trials, an effect which should be studied and addressed in animal studies (168;170-173). Randomization involves taking proactive measures in assigning subjects to a treatment or control group in studies testing the efficacy of a potential treatment drug versus a placebo control or potential treatment drug versus a pre-existing treatment (167;173). The systematic randomization of subjects or animals minimizes allocation or selection bias by eliminating influences from unknown or known prognostic variables (like the hemodynamics in septic and nonseptic animals) which may influence the response to treatment, mortality, or outcome, unduly producing biased results in a study (166;170-172). Randomization also ensures that protocols and procedures are conducted consistently and systematically in treatment and control groups. Elements of animal studies where randomization may offer an efficacious benefit include the procedure to induce sepsis (preventing unintentional bias by variations in sepsis severity), treatment (consistent timing, route, and method of administering treatments or controls), monitoring of subjects following sepsis induction and treatment, and resuscitation procedures of the study groups (169;173).

Although various methods of randomization have been established and recommended for different clinical trials, consensus and literature on randomization in pre-clinical and experimental studies are lacking. Simple randomization methods such as the "repeated fair coin-tossing" have been recommended for large clinical studies ($n > 200$), but are inadequate for studies with small samples sizes like experimental animal studies (167;173). Restricted randomization methods like permutated-block randomization where allocation ratios are used to specify the number of subjects in each group (or ratio of subjects in one treatment group to another) (170-172) successfully address this issue, but restricted randomization methods have not been validated in animal studies. Given that the risk of allocation and selection bias have been shown to unduly influence the results of clinical studies and that randomization and blinding have successfully minimized these risks, it is crucial that the same level of criticism and caution be applied in the experimental design and conducting of experimental studies by addressing these risks (167;170-173).

7.4.4. Blinding

In addition to randomization, blinding of investigators also minimizes the risk of bias at many levels of the preclinical study. Blinding decreases differential treatment of animals in the study groups (more intensive monitoring or closer supervision of one treatment group over another) to unintentional biases in analyzing data and adjudicating outcomes (5;166). For instance, markers of disease severity or mortality outcome such as organ histology may be interpreted in a biased manner in an untreated control group versus the treated group if the investigator is not blinded to the treatment received (169). Likewise, marginal findings may be analyzed in a biased manner when interpretation of the outcome marker is subjective. In studies where substitute endpoints are used (such is the case when mortality is not used as endpoints for ethical reasons), blinding reduces the chance of experimenters prolonging the time to endpoint (in an attempt to establish a therapeutic effect when one does not exist) or decreasing the time to a substitute endpoint or even excluding animals (if unexpected results are observed in either treatment or control groups) (165;167;170;172). Blinding of the investigator to the treatment or control substance received minimizes the risk of experimenter bias.

7.4.5. Analytic plan

The analytic plan should be described in detail such that the procedure is reproducible by others and variations in models used by other investigators can be appreciated (e.g. size and number of enterotomies and location of ligature in a CLP model) when comparing various preclinical studies (5). Primary and secondary endpoints of the study should be clearly outlined, whether they are to determine the efficacy of a potential therapy, improve prognosis, or diagnosis of sepsis. If the primary objective is to investigate a potential therapy, endpoints and markers of outcome of the animal study should also be clinically relevant (166;173;174). The measurement of outcome should also appropriately reflect markers relevant to the potential therapy being tested. For example, the coagulant state of the animals should be measured if the drug being tested acts via its anticoagulant effects. While mortality is the most informative marker of outcome in clinical sepsis, organ health and dysfunction are useful surrogate markers of outcome due to their relevance to clinical sepsis (173).

7.4.6. Power calculations

In studies elucidating the therapeutic potential of a compound, power calculations can be used to reliably determine the sample size required to show that an effect is associated with treatment as well as exclude an effect when none exists (5). The magnitude of a treatment effect is most commonly expressed as the relative risk reduction (RRR, RRR = [1-(X/Y)] x 100 where Y is the proportion of animals that expired in the treatment group and X is the proportion of animals that expired in the control group (175). Given that mortality (and therefore the power of the study) will be altered should any subjects be excluded, the exclusion of subjects should only occur if subjects meet pre-established criteria.

7.4.7. Exclusion criteria

As in clinical studies, conditions under which subjects will be excluded should be clearly outlined before the animal study (169). The study should account for all animals, including those that do not complete the experimental protocol and observations and reasons for excluded animals should be appropriately documented. This is important to determine whether prognosis independent of the treatment may in actuality be related to the exclusion of certain subjects (5;174). Data should also be recorded and kept for subjects excluded from the study for any post-hoc analyses, standards which are expected of clinical studies.

7.4.8. Animal welfare

Many animal ethics and welfare committees are preventing the use of mortality as an endpoint and encouraging the use of surrogate markers of death. However, investigators and funding agencies are critical of such studies which do not use changes in mortality as a measure of therapeutic efficacy. There is currently no consensus nor scientific validation of clinically relevant, reliable substitute markers of death in septic animals (5;174). Moreover, there is concern over the use of analgesics and anaesthetics which have been shown to interfere with components in the natural progression of sepsis and even exert protective, anti-inflammatory effects. For example, *in vivo* and *in vitro* studies have elucidated that pentobarbital significantly reduces the LPS-induced inflammatory response by suppressing TNF α mRNA and protein expression by NF-κB and p38 MAP kinase (176). The concern over analgesics and anaesthetics Interfering with disease progression and treatment response may cause some reluctance towards the appropriate use of analgesics and anaesthetics in preclinical studies. Although scientific concern is warranted, these findings highlight the importance of considering drug effects and incorporating them into the experimental design, as it should be noted that analgesics and anaesthetics are clinically relevant to treatment and management of the septic patient, and from the standpoint of relevance (not to mention from an ethical standpoint), should not be excluded in animal studies. To improve clinical relevance and translation of studies utilizing experimental models, the appropriate use of these drugs with further understanding of their effects should be integrated rather than avoided in preclinical studies of sepsis (5;165).

7.5. Recommendations for a thorough validation of therapeutic efficacy in preclinical studies

Given the potential limitations of preclinical studies when designing preclinical studies with potential clinical applications, one should take a critical approach when validating the therapeutic efficacy of a potential drug in preclinical studies. Recommendations for conducting preclinical studies with clinical applications will be provided with the intent to address the concerns raised previously. Literature by Marshall et al. suggest that early proof

of concept studies should 1) delineate a potential pathological role for the target in question by quantifying its increases in a simple acute model like endotoxemia or bacteremia (LPS or E. coli challenge) and 2) demonstrate that attenuating such target may improve outcome or offer some therapeutic benefit by a measurable decrease in harmful effects of the initial challenge or insult (5;165;174). If a proof of concept can be established, time course studies should be conducted to determine if the potential treatment offers therapeutic effects if administered before, during the progression of, or after the insult in both simple acute and complex models (e.g. LPS challenge and CLP). Experimental methodology and design of further studies should consider the results of studies on targets with similar biological or pathophysiological effects. Any adverse effects of the intervention, like impairment of the host's natural anti-microbial immune function or further dysregulation of the coagulant state should be determined in a high-risk model using live organisms as the challenges. (5;174) Moreover, the systemic physiological and biological responses to the therapeutic intervention (e.g. cardiac output, oxygenation, glomerular filtration) should be determined in a large animal model, given the limitations of small rodent models of sepsis. Interventions used in the management of clinical sepsis such as fluid resuscitation, ventilatory support, and hemodynamic monitoring, can be incorporated into the study design of preclinical trials using large animal models (5).

8. Goals for the future of experimental sepsis research

Goals for the future use of experimental sepsis models ought to focus on improving the relevance, translation, and applicability of results and inferences from animal studies to what is observed clinically. This requires consideration of the limitations of current sepsis models described such as considering age, gender, genetic background, nutritional status, and the environment of the housing facility, incorporating cormorbidities and supportive therapies which may be clinically relevant, and finally addressing the risk of bias by randomization and blinding methods in preclinical studies of sepsis.

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