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Insulin Therapy for Hyperglycemia in Neonatal Sepsis Using a Preterm Mouse Model

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Abstract

BACKGROUND: Stress-induced hyperglycemia is a frequent complication of neonatal sepsis. Hyperglycemia induces oxidative stress and immunosuppression. We investigated the glucose kinetics and effect of insulin administration during stress-induced hyperglycemia in a neonatal sepsis mouse model.

METHODS: A stock cecal slurry (CS) solution was prepared from adult cecums and 3.0 mg of CS/g (LD_{40}) was administered intraperitoneally to 4-day-old FVB mouse pups. Blood glucose (BG) levels were measured at 1.5, 3, 6, and 9 h post-sepsis induction and compared with basal levels. Two different doses of ultrafast-acting insulin were administered subcutaneously, and BG levels and survival rates were monitored.

RESULTS: BG levels were significantly higher than that of baseline levels with a peak at 3 h, which progressively decreased from 6 to 9 h post-sepsis induction. Insulin treatment reduced post-sepsis induced hyperglycemia at 1.5 and 3 h. The mortality rate of CS-only pups (39%) was similar to that of CS+1 U/kg insulin pups (60%). However, the mortality rate of CS+5 U/kg insulin pups (82%) was significantly higher than that of CS-only pups.

CONCLUSION: Marked hyperglycemia was induced immediately after post-sepsis induction, and the high-dose insulin treatment increased mortality post-induction. Therefore, stress-induced hyperglycemia could be a physiological and protective response for preterm sepsis, and aggressive treatment of this hyperglycemia might be contraindicated.

Keywords: Insulin therapy, hyperglycemia, neonatal peritoneal sepsis, mouse model

Abbreviations

GLUT; glucose transporter

BG; blood glucose

CLP; Cecal Ligation and Puncture

CS; cecal slurry

CFUs; colony forming units

bw; bodyweight

LPS; Lipopolysaccharide

ROS; reactive oxygen species

Introduction

Neonatal peritoneal sepsis is secondary to gastrointestinal perforation and is a life-threatening condition with poor prognosis, which requires a multidisciplinary approach for treatment [1]. Septic stress activates the hypothalamus–pituitary–adrenal systems, sympathetic nervous system, and immune system, resulting in gluconeogenesis, glycogenolysis, and insulin resistance in each tissue, and decreased insulin secretion in the pancreas [2]. The neonatal period is a transitional period from when the glucose transporter-1 (GLUT-1) is predominantly expressed in each tissue to the state where GLUT-1 to -4, which is specific to each tissue, is expressed [3]. Thus, both insulin secretion ability and tissue insulin responsiveness to change in blood glucose (BG) levels is low [4]. Stress-induced hyperglycemia is frequently associated with neonatal sepsis [5, 6]. Septic stress and stress-induced hyperglycemia can induce oxidative stress, inflammatory cytokines, immunosuppression, vasospasm, and thrombus formation, resulting in aggravation of the septic condition [2, 7, 8]. In neonates, hyperglycemia increases acute mortality and causes long-term neurological sequelae risk [9, 10].

In adult critical care, insulin has anti-inflammatory, anti-oxidative, vasodilative, and platelet aggregation inhibitory effects, in addition to an anti-hyperglycemic function [11]. Barkhausen et al. [12] suggested that insulin had a direct anti-inflammatory effect that was independent of the modulation of BG levels, using the murine 2-hit (femoral fracture and Cecal Ligation and Puncture [CLP]) model. By examining the hippocampus and frontal cortex from adult septic mice, Sonneville et al. [13] clarified that the percentage of damaged neurons was significantly higher in the brains of hyperglycemic ill mice than those of healthy mice, and that these neuropathological changes were partially prevented by BG control via insulin treatment. In addition, Delile et al. [14] reported that the modulation of CLP-induced insulin resistance via protein tyrosine phosphatase 1B blockade resulted in an improvement of sepsis-induced

endothelial dysfunction/impaired nitric oxide production. Thus, glycemic control by insulin therapy might be an option for improving outcomes in septic patients. Recently, several guidelines and consensus statements have recommended insulin therapy for marked hyperglycemia in critically ill patients, including sepsis [15, 16]. However, only a few studies have reported the protective effects of insulin therapy for neonatal hyperglycemia, and the efficacy and safety of aggressive insulin therapy for critically ill neonates remains unclear [17].

In the present study, we examined the effect of insulin administration in a preterm sepsis mouse model.

Materials and Methods

Animals

Adult FVB/NJcl breeders were obtained from CLEA Japan, Inc. (Tokyo, Japan) and were fed a standard rodent diet and water ad libitum. All pups remained with their mothers throughout the entire study. Pups were individually randomized within each litter to eliminate all litter bias effects. In addition, we used at least three different litters for the survival experiment. All studies were performed in accordance with the National Institute of Health guidelines and with the approval of the Institutional Animal Care and Use Committee (P170611).

Bacterial viability of the cecal slurry (CS) stock

As described previously [18], a single CS stock solution was prepared from adult cecums and stored at −80°C in 1 mL aliquots until subsequent use. For each sepsis induction, an aliquot of CS stock solution was thawed at room temperature (25-30°C) and 50 μL was plated onto 1.5% agar containing brain/heart infusion broth [19]. Agar plates were then incubated at 37°C for 24 h and

colony forming units (CFUs) were counted. For the entire study, the mean CFU count was 1.1 ± 0.3 × 10^4 CFU/mL (n = 20).

Sepsis induction

To induce sepsis, 4-day-old mouse pups, which are immunologically equivalent to human preterm infants [18, 20], were given various doses of CS intraperitoneally, and then closely monitored daily for health and survival for up to 7 days. The survival rates were 100% (n = 10 pups), 83.3% (n = 18), 61.1% (n = 18), and 41.1% (n = 17) for CS doses of 1.0, 2.0, 3.0, and 4.0 mg/g bodyweight (bw), respectively (**Fig. 1**). For the following experiment, we used the 3.0 mg/g bw (LD_{40}) CS dose for sepsis induction.

BG measurement

BG levels were measured from whole blood using an ABL-90 FLEX analyzer (Radiometer Medical ApS, Brønshøj, Denmark). As a normal control, 4-day-old mice without sepsis induction were euthanized for blood collection and BG levels were measured. As a sepsis group (CS group), 4-day-old mice were sacrificed at 1.5, 3, 6, and 9 h post-sepsis induction for blood collection and BG levels were measured. To determine the timing of the BG peak, BG levels were compared between the different time points within the CS group, and then the BG levels at each time point were compared with control values.

Insulin treatment

To determine the insulin doses used in the study, normal saline (vehicle) or various doses (1–10 U/kg) of ultrafast-acting insulin analog (NovoRapid@, Novo Nordisk Pharma Ltd, Tokyo, Japan) were administered subcutaneously to 4-day-old pups at the time of sepsis induction, and BG levels 3 h post-sepsis induction were measured. To assess the effects of insulin therapy on sepsis, two different doses of insulin (1 or 5 U/kg) were administered subcutaneously at the time of

sepsis induction, and the BG levels at the timepoints described above, blood lactate levels at 3, 6, and 9 h and bw change at 24 h post-sepsis induction, and survival rates were monitored.

Statistical analyses

Statistical analyses were performed using the log-rank test for survival curves, Kruskal-Wallis test for comparison of different time points within the group, and the Mann–Whitney test for comparisons of two groups. Differences were considered statistically significant at p values less than 0.05.

Results

Transition of BG levels in the preterm sepsis mouse model

The BG level of the 4-day-old mice without sepsis induction was 72 ± 14 mg/d (basal levels, n = 13). The BG levels significantly changed post-sepsis induction ($p = 0.0001$). The BG level increased significantly at 1.5 h (253 ± 53 mg/dL, n = 7, $p < 0.0001$ vs basal levels) and reached a peak at 3 h (412 ± 46 mg/dL, n = 9, $p < 0.0001$ vs basal levels) post-sepsis induction, and then progressively decreased from 6 h (203 ± 168 mg/dL, n = 6, $p = 0.27$ vs basal levels) to 9 h post-sepsis induction (80 ± 115 mg/dL, n = 10, $p = 0.01$ vs basal levels, **Fig. 2**).

Effect of insulin on the transition of BG levels in the preterm sepsis mouse model

In the 1 U/kg bw of insulin-treated (CS+Ins1) pups, the BG levels increased significantly at 1.5 h (146 ± 37 mg/dL, n = 7, $p < 0.001$ vs basal levels) and reached a peak at 3 h (277 ± 134 mg/dL, n = 5, $p < 0.001$ vs basal levels) and 6 h (279 ± 148 mg/dL, n = 13, $p = 0.11$ vs basal levels) post-sepsis induction, and then decreased to 9 h post-sepsis induction (115 ± 154 mg/dL, n = 10, $p = 0.11$ vs basal levels, **Fig. 2**). In the 5 U/kg bw of insulin-treated (CS+Ins5) pups, the BG levels increased significantly at 1.5 h (115 ± 38 mg/dL, n = 7, $p < 0.001$ vs basal levels), reached a peak at 3 h post-sepsis induction (159 ± 24 mg/dL, n = 5, $p < 0.001$ vs basal levels), and then

progressively decreased from 6 h (84 ± 49 mg/dL, n = 13, p = 0.91 vs basal levels) to 9 h post-sepsis induction (41 ± 25 mg/dL, n = 9, p < 0.01 vs basal levels, **Fig. 2**). When compared to CS-only treated pups at each timepoint, the BG levels of CS+Ins1 treated pups were significantly lower at 1.5 and 3 h post-sepsis induction (p < 0.001 and p = 0.03, respectively); however, there were no significant differences at 6 and 9 h post-sepsis induction (p = 0.28 and p = 0.81, respectively). Similarly, the BG levels of CS+Ins5 treated pups were significantly lower at 1.5 and 3 h post-sepsis induction (p < 0.001 and p < 0.005, respectively); however, there were no significant differences at 6 and 9 h post-sepsis induction (p = 0.27 and p = 0.96, respectively).

Effect of insulin on bodyweight change and mortality in the preterm sepsis mouse model

When comparing the bw gain at 24 h post-sepsis induction of surviving pups only, the bw of CS-only treated groups increased (1.6 ± 8.7%, n=16), and those of CS+Ins1 and CS+Ins5 treated groups decreased (-0.9 ± 6.5%, n=9, and -1.6 ± 3.2%, n=4, respectively), despite no significant differences among the groups. The mortality rate of the CS-only treated pups (39%, n = 18) was similar to that of the CS+Ins1 treated pups (60%, n = 10, p = 0.28). However, the mortality rate of the CS+Ins5 treated pups (82%, n = 11) was significantly higher than that of the CS-only treated pups (p < 0.01, **Fig. 3**).

Effect of insulin on blood lactate levels

At 3 h post-sepsis induction, lactate levels were significantly higher in pups of the CS-only group compared with those of the CS+Ins1 and CS+Ins5 groups (p<0.01 and p<0.05, respectively, Table 1). Although the lactate levels of the CS-only group were still higher than those of the other groups at 6 and 9 h post sepsis induction, there was no statistically significant difference among the groups.

Discussion

In the present study, two important observations were noted. First, the non-surgical preterm sepsis mouse model exhibited clear stress-induced hyperglycemia that peaked at 3 h post-sepsis induction. Second, insulin administration to this model suppressed hyperglycemia; however, glycemic control did not contribute to the improvement of mortality rates.

A 4-day-old preterm sepsis mouse model, which is immunologically equivalent to a human preterm infant, exhibited hyperglycemia peaking at 3 h after intraperitoneal CS administration, after which BG levels gradually decreased. In terms of sepsis-induced hyperglycemia, Zeng and colleagues [21] reported for adult Lipopolysaccharide (LPS)-induced septic rats that BG levels increased significantly at 2 and 6 h post-sepsis induction. Yamashita et al. [22] reported that a CLP operation led to hyperglycemia that lasted longer (18 h) than that after a sham operation (6 h) in their adult septic mouse model. Regarding the neonatal sepsis animal models, Bhola et al. [23] reported that 10-day-old suckling rats of the LPS-induced shock model demonstrated hypoglycemia at 8 h post-sepsis induction. Zeller et al. [24] compared the temporal response to endotoxin of 0-, 10-, and 28-day-old rats, and reported that BG levels increased in the high mortality groups after 2 h in the 0- and 10-day-old rats, and after 1 h in the 28-day-old rats, suggesting that insulin response to LPS differed with animal age. In the present study, we elucidated a detailed BG transition in the youngest sepsis neonatal mouse model, and our model might be suitable to study neonatal stress-induced hyperglycemia because of the high reproducibility of changes of BG levels with a clear peak soon after post-sepsis induction.

Although the administration of insulin doses of 1 and 5 U/kg bw successfully suppressed BG increase at 3 h post-sepsis induction, the sepsis mortality of the insulin-treated groups did not improve. Similarly, in adult clinical trials, when strict glycemic control was applied to stress the hyperglycemia of critically ill patients, the mortality rate was higher than those treated with a

conventional glycemic control [25]. Tiruvoipati et al. [26] reported that in septic shock, the mortality rate was lower in the group exhibiting stress-induced hyperglycemia than in the normal BG group, which might suggest that insulin therapy for stress-induced hyperglycemia is effective only when there is an abnormally high BG level. Based on the above studies, the current guideline of adult sepsis states that insulin therapy is recommended when the BG level is above 180 mg/dL, and the BG level should be kept at greater than 110 mg/dL during treatment [15].

Insulin therapy has been attempted in ill neonates as well as adults for hyperglycemia; however, there are no clinical trials that provide concrete evidence for its benefits [17]. Several authors have reported that for very low birth weight infants with hyperglycemia, insulin therapy has been effective in maintaining nutritional status without increased mortality and complications [27, 28]. In contrast, Beardsall et al. [29] reported that sustained intravenous insulin administration with glucose to extremely low birth weight infants resulted in increased mortality despite the low frequency of hyperglycemia or weight loss. In addition, in a randomized controlled trial comparing tight BG control (72–108 mg/dL) and standard BG control (144–180 mg/dL) by continuous insulin administration for extremely low birth weight infants with hyperglycemia, the risk of hypoglycemia was higher in the tight control than that of the standard group, despite no difference in mortality and morbidities [30]. Thus, the safety and efficacy of insulin therapy for newborns is still controversial.

Stress-induced hyperglycemia in severe illness promotes glucose uptake into immune cells and tissues with decreased blood flow by the osmotic pressure gradient [31, 32]. In adults, GLUT expression and glucose utilization are increased in tissues during stress, which take in sugars in an insulin-independent manner such as the brain and blood cells, whereas GLUT expression and glucose utilization are decreased in peripheral tissues, such as skeletal muscle and adipose tissues. Therefore, stress-induced hyperglycemia might be an inherently essential phenomenon for energy

to support brain activity and immune response [32]. Thus, the reason insulin administration did not increase the survival rate of mice in the present study might be that the initial suppression of glycemic elevation inhibited the intrinsic immune response and brain protection caused by stress-induced hyperglycemia against septic insults.

In adult sepsis, phagocytes and endothelial cells produce reactive oxygen species (ROS) to eliminate pathogens [33-35]. Stress-induced hyperglycemia also increases ROS production from phagocytes and vascular endothelial cells [11]. Thus, sepsis-induced oxidative stress causes direct cell damage and is associated with the exacerbation of the pathological condition via hemodynamic deterioration [36]. Kakita et al. [37] reported that ROS peaked at 1 h post-sepsis induction in their newborn swine sepsis model generated by CLP, and there was a clear difference in the timing of peak ROS levels from the adult septic rats reported by Ritter et al. [38], which peaked at 37–48 h post-sepsis induction. Thus, we hypothesized that there are different oxidative stress dynamics in adult and neonatal sepsis, which could be the mechanisms of the unfavorable outcome of insulin therapy in the present study.

The limitation of our present study is that, in clinical practice, insulin therapy is performed in combination with the careful prevention of hypoglycemia including frequent BG measurements and intravenous glucose administration; however, in the present study using a neonatal mouse model, these adjuvant treatments were not applied owing to technical difficulties of blood sampling and continuous infusion under survival. It is therefore unclear whether the present results indicate that the suppression of hyperglycemia was truly ineffective, and it is possible that the adverse effects of unexpected hypoglycemia diminished the positive effect of insulin therapy. In this study, insulin therapy had a positive effect on peripheral circulation indicated by blood lactate levels at least in the early phase of sepsis.

Thus, to clarify the effect of strict BG control during sepsis in neonates, further studies using larger neonatal animal models that can be used together with frequent BG measurement and intravenous infusion are required. In addition, because many of the adverse effects of hyperglycemia are mediated via the production of oxidative stress, it is important to study the dynamics of oxidative stress, as well as infection-related markers, including white blood cells to elucidate the reason that BG control via insulin treatment failed in our neonatal septic mouse model.

We found that hyperglycemia was induced immediately post-sepsis induction and the high dose insulin treatment increased mortality post-induction. Therefore, stress-induced hyperglycemia could be a physiological and protective response for preterm sepsis, and aggressive treatment of this hyperglycemia might be contraindicated.

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Disclosure

The authors declare no conflict of interest. This study did not require IRB review because it did not involve human subjects.

Author contributions

K.F. and S.O. contributed to the conception and design of the study; H.M., K.F., S.O., K.N., M.A., and T.I. performed the experiments; H.M. and K.F. performed statistical analysis and drafted the

manuscript; Y.O., K.M., K.I., and Y.B. reviewed the manuscript and supervised the whole study process. All authors read and approved the final manuscript.

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Figure Legends

Fig. 1 Survival curves. Kaplan-Meier survival plots of 4-day-old pups given various doses of cecal slurry intraperitoneally: 1.0 (□, n = 10), 2.0 (△, n = 18), 3.0 (○, n = 18), or 4.0 (◇, n = 17) mg/g. A significant dose-dependent effect on mortality with an LD_{40} of 3.0 mg/g bw was found. Pups from at least three different litters were used in each group.

Fig. 2 BG level changes in the preterm sepsis mouse model. Data are shown as scatter plot of whole data with mean bar. CS-only (black circle): cecal slurry (CS) 3.0 mg/kg bodyweight (bw) treated group. CS+Ins1 (dark grey square): CS 3.0 mg/kg bw + Insulin 1 U/kg bw treated group. CS+Ins5 (light grey triangle): CS 3.0 mg/kg bw+ Insulin 5 U/kg bw treated group. CS+Ins10 (white rhombus): CS 3.0 mg/kg bw+ Insulin 10 U/kg bw treated group. * $p < 0.05$, ** $p < 0.01$, vs basal levels. # $p < 0.05$, ## $p < 0.01$ vs CS-only group at each timepoint.

Fig. 3 Effect of insulin on the mortality in the preterm sepsis mouse model. The mortality rate of CS-only treated pups (○, n = 18) was similar to that of CS+Ins1 pups (□, n = 10). However, the mortality rate of CS+Ins5 pups (■, n = 11) was significantly higher than that of CS-pups ($p < 0.01$ vs CS-only treated pups, log-rank-test). Pups from at least three different litters were used in each group. CS, cecal slurry; Ins, Insulin

Table. 1 Lactate level changes post-sepsis induction

Lac (mmol/L)	CS-only	CS+Ins1	CS+Ins5
3h	7.0±0.7 (5)	4.8±0.7## (5)	5.4±0.7# (5)
6h	6.8±1.5 (6)	5.2±1.2 (4)	4.9±1.1 (4)
9h	5.3±0.6 (3)	4.3±1.0 (5)	4.6±2.8 (5)

Data are shown mean ± standard deviation (number).

CS-only: cecal slurry (CS) 3.0 mg/kg bodyweight (bw) treated group

CS+Ins1: CS 3.0 mg/kg bw + Insulin 1 U/kg bw treated group

CS+Ins5: CS 3.0 mg/kg bw+ Insulin 5 U/kg bw treated group

# $p < 0.05$, ## $p < 0.01$ vs CS-only group at each timepoint.

Figure 1

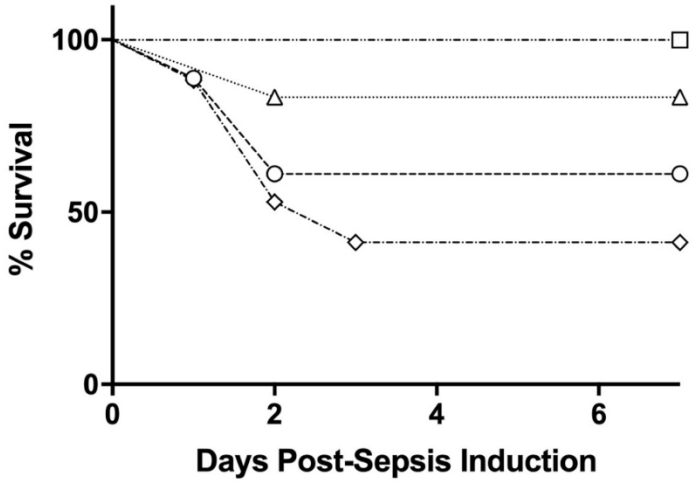

Fig. 1 Survival curves. Kaplan-Meier survival plots of 4-day-old pups given various doses of cecal slurry intraperitoneally: 1.0 (□, n = 10), 2.0 (△, n = 18), 3.0 (○, n = 18), or 4.0 (◇, n = 17) mg/g. A significant dose-dependent effect on mortality with an LD40 of 3.0 mg/g bw was found. Pups from at least three different litters were used in each group.

190x254mm (300 x 300 DPI)

Figure 2

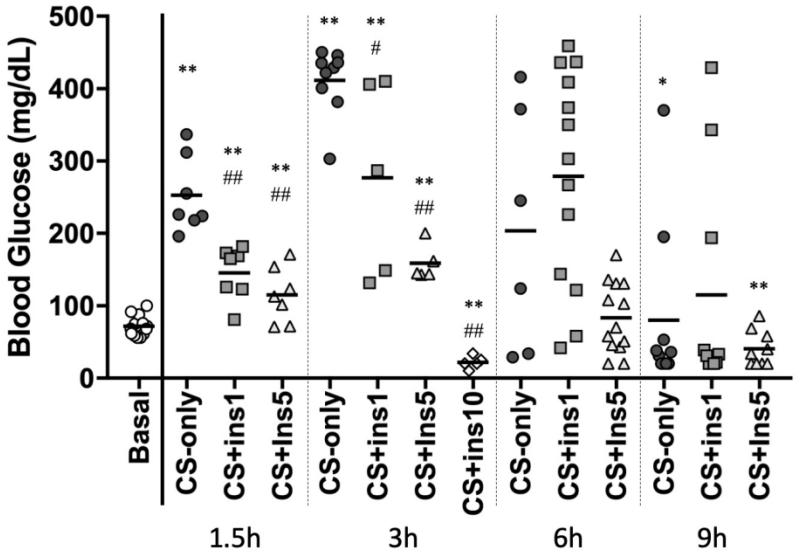

Fig. 2 BG level changes in the preterm sepsis mouse model. Data are shown as scatter plot of whole data with mean bar. CS-only (black circle): cecal slurry (CS) 3.0 mg/kg bodyweight (bw) treated group. CS+Ins1 (dark grey square): CS 3.0 mg/kg bw + Insulin 1 U/kg bw treated group. CS+Ins5 (light grey triangle): CS 3.0 mg/kg bw+ Insulin 5 U/kg bw treated group. CS+Ins10 (white rhombus): CS 3.0 mg/kg bw+ Insulin 10 U/kg bw treated group. * $p < 0.05$, ** $p < 0.01$, vs basal levels. # $p < 0.05$, ## $p < 0.01$ vs CS-only group at each timepoint.

190x254mm (200 x 200 DPI)

Figure 3

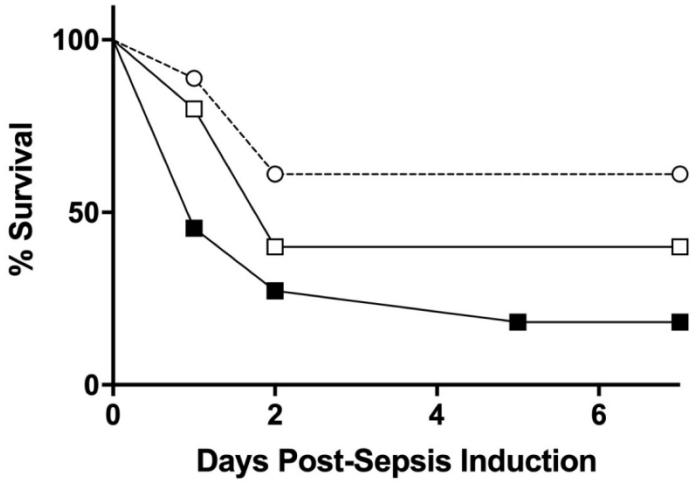

Fig. 3 Effect of insulin on the mortality in the preterm sepsis mouse model. The mortality rate of CS-only treated pups (○, n = 18) was similar to that of CS+Ins1 pups (□, n = 10). However, the mortality rate of CS+Ins5 pups (■, n = 11) was significantly higher than that of CS-pups ($p < 0.01$ vs CS-only treated pups, log-rank-test). Pups from at least three different litters were used in each group. CS, cecal slurry; Ins, Insulin

190x254mm (200 x 200 DPI)