ASSESSMENT OF INTENSIVE CARE UNIT-ACQUIRED WEAKNESS IN YOUNG AND OLD MICE: AN E. coli SEPTIC PERITONITIS MODEL

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ABSTRACT: Introduction: There are few reports of in vivo muscle strength measurements in animal models of ICU-acquired weakness (ICU-AW). In this study we investigated whether the Escherichia coli (E. coli) septic peritonitis mouse model may serve as an ICU-AW model using in vivo strength measurements and myosin/actin assays, and whether development of ICU-AW is age-dependent in this model. Methods: Young and old mice were injected intraperitoneally with E. coli and treated with ceftriaxone. Forelimb grip strength was measured at multiple time points, and the myosin/actin ratio in muscle was determined. Results: E. coli administration was not associated with grip strength decrease, neither in young nor in old mice. In old mice, the myosin/actin ratio was lower in E. coli mice at t = 48 h and higher at t = 72 h compared with controls. Conclusions: This E. coli septic peritonitis mouse model did not induce decreased grip strength. In its current form, it seems unsuitable as a model for ICU-AW.

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INTRODUCTION

Intensive care unit-acquired weakness (ICU-AW) is a frequent complication of critical illness, which causes long-term impairments in physical function.1,2 ICU-AW is caused by critical illness myopathy (CIM), critical illness polyneuropathy (CIP), or a combination of both (CINM).3 The most prominent characteristic and prerequisite for the diagnosis of ICU-AW is decreased muscle strength as measured by muscle strength testing in awake and responsive patients.1

Several sepsis animal models have been used to study ICU-AW, as sepsis is the main risk factor for development of ICU-AW.2 However, the models used so far have severe limitations. First, in vivo quantitative muscle strength measurements in awake mice have been performed infrequently. Instead, other markers for muscle function have been used, like electromyography or contractility measurements, which require anesthesia.4–6 Moreover, animal models often use a short period of sepsis, which is not comparable to the chronic sepsis seen in ICU-AW patients.7 Models of long-lasting sepsis, such as the porcine model of acute quadriplegic myopathy, are very expensive and time-consuming.7 Finally, animals used in experiments are often young, whereas increasing age has been described as a risk factor for ICU-AW.8,9 Therefore, a new animal model to study ICU-AW is needed to ensure more reliable translation of results from animal experiments to the bedside. In such a new model, animals with sepsis should show a clinically significant decline in muscle strength over time, and animals of older age should be studied. Because a selective loss of myosin filaments is seen in patients with ICU-AW caused by CIM or CINM, the myosin/actin ratio can also be used to characterize a new animal model.10

Intra-abdominal infections are an important cause of human sepsis, and Escherichia coli (E. coli) bacteria are frequently involved.11 In this study, we used the E. coli septic peritonitis mouse model, a well-established animal model for sepsis.12,13

The objectives of this study were to investigate: (1) whether the E. coli septic peritonitis model induces ICU-AW using both in vivo strength measurements and in vitro myosin/actin assays and (2) whether development of decline in muscle strength is age-dependent in this model.

MATERIALS AND METHODS

Animals and Ethics Statement. A total of 108 male, specific pathogen-free, C57BL/6J mice were obtained from Charles River; 54 mice were 8 weeks old (young mice), and 54 were 13 months old (old mice). Mice were housed in groups of 5 to 6 in individually ventilated cages for at least 2 weeks before the start of the experiment. All experiments conformed to the Dutch Experiments on Animals Act for the care and use of animals and were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center,
Amsterdam, the Netherlands (permit number: 102639). Food and water were available ad libitum, and a 12:12 h light–dark cycle was retained. This manuscript was drafted in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.14

**Table 1. Experimental groups.**

| Group        | t = 0          | t = 12         | t = 24         | t = 48         | t = 72         |
|--------------|----------------|----------------|----------------|----------------|----------------|
| E+A group    | E. coli        | Ceftriaxone    | Ceftriaxone    | Sacrificed     | Sacrificed     |
| n = 30 young | n = 15 young   | n = 15 young   | n = 14 old*    | n = 14 old*    | n = 14 old*    |
| n = 30 old   | n = 15 old     | n = 15 old     | n = 14 old*    | n = 14 old*    | n = 14 old*    |
| C+A group    | Saline         | Ceftriaxone    | Ceftriaxone    | Sacrificed     | Sacrificed     |
| n = 12 young | n = 6 young    | n = 6 young    | n = 6 old      | n = 6 old      | n = 6 old      |
| n = 12 old   | n = 6 old      | n = 6 old      | n = 6 old      | n = 6 old      | n = 6 old      |
| C group      | Saline         | Saline         | Saline         | Sacrificed     | Sacrificed     |
| n = 12 young | n = 6 young    | n = 6 young    | n = 6 old      | n = 6 old      | n = 6 old      |
| n = 12 old   | n = 6 old      | n = 6 old      | n = 6 old      | n = 6 old      | n = 6 old      |

*One old mouse from the E+A group died before the end of the experiment. E+A, E. coli and antibiotics; C+A, control and antibiotics; C, control.

**Induction of E. coli Peritonitis and Treatment with Antibiotics.** *E. coli* (O18:K1) was cultured in Luria-Bertani medium at 37°C to mid-log phase in 1 h, 45 min. The amount of bacteria in the culture was estimated by measuring the A600 in a spectrophotometer. Viable *E. coli* were harvested by centrifugation at 3,000 rpm for 10 min and washed twice with pyrogen-free sterile isotonic saline. The bacteria were diluted to a final concentration of 1 × 10^4 colony forming units (CFU) /200 µl (range, 0.8 × 10^4 - 1.2 × 10^4 CFU) in pyrogen-free sterile isotonic saline. Experiments were performed in 2 different rounds (first round, 22 young mice and 22 old mice; second round, 32 young mice and 32 old mice). Serial 10-fold dilutions of the bacterial inoculum were plated on blood agar plates and incubated overnight at 37°C to verify the amount of viable bacteria injected.

Without antibiotic treatment, 80% of mice in this model die within 48 h.13 To improve survival and prolong the duration of the model, enabling repeated muscle strength measurements over time, mice received antibiotic treatment with ceftriaxone (Fresenius Kabi, Den Bosch, the Netherlands) 10 µl/g body weight (20 mg/kg).

**Experimental Procedure.** Young and old mice were assigned to 3 groups (each cage was randomly assigned to 1 of the groups): *E. coli* and antibiotics (E+A group, 30 young mice and 30 old mice), control and antibiotics (C+A group, 12 young mice and 12 old mice), and control (C group, 12 young mice and 12 old mice) (Table 1).

At the start of the experiment (t = 0), E+A mice were injected intraperitoneally with 200 µl of the bacterial inoculum; the C+A group and C group were injected intraperitoneally with 200 µl of pyrogen-free sterile isotonic saline. At t = 12 and t = 24 h after injection, E+A and C+A mice received an intraperitoneal injection with ceftriaxone. At these time points mice of the C group received saline (10 µl/g body weight). Body weight was measured at baseline, at t = 12, t = 24, t = 48, and t = 72 h. Half of the animals in each group were euthanized at t = 48 h and the other half at t = 72 h. Animals were euthanized by an intraperitoneal injection of a mix of ketamine 190 mg/kg and medetomidine 0.3 mg/kg followed by heart puncture and blood aspiration. Blood, spleen, and liver were harvested for determination of bacterial outgrowth. Tibialis anterior muscles were removed, snap frozen in liquid nitrogen, and stored at −80°C.

**Grip Strength Testing.** At t = 0, t = 12, t = 24, and t = 48 (at t = 48 half of the animals), forelimb grip strength was measured using a grip strength meter with metal grid (Bioseb, France). Mice were held at the base of the tail above the top of the grid. After holding the grid, they were pulled backward horizontally until the grip was released. Maximal force developed by the animal was recorded by the grip strength meter. At each time point, 3 grip strength measurements were taken, and the average result was used for analysis. Grip strength was normalized for concomitant body weight.15

**Bacterial Outgrowth.** Spleen and liver were homogenized in 4 volumes of sterile saline with a tissue homogenizer.

Serial 10-fold dilutions of blood, liver, and spleen homogenates were plated on blood agar plates, and bacteria were allowed to grow overnight at 37°C.

**Whole Muscle Homogenate.** Frozen muscle samples of E+A and C+A mice were pulverized with a precooled mortar and hammer. The pulverized muscle was then suspended in a vial with 500 µl of lysis buffer [(5 mM Sigma 7-9, 50 mM NaF, 2 mM
Na$_3$VO$_4$, 2 mM ethyleneglycol tetraacetic acid, distilled water, with protease inhibitor mix (aprotinin, leupeptin, and pepsatin in Tris-HCl solution), and dithiothreitol. Tissue was homogenized by use of a tissue homogenizer. During the procedure, samples were kept on ice. Samples were centrifuged (1 min at 300 $g$, 4°C), and the supernatant was used as whole muscle homogenate and stored at –80°C.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis and Western Blot Myosin/Actin Ratio. To investigate if selective myosin loss is found in this model, we assessed the myosin/actin ratio in muscle tissue of E$_1$A as compared to C+$A$. Total protein content of whole muscle homogenate was determined using the Lowry protein assay.$^{16}$ Samples were normalized to an equal total protein concentration. Equal volumes were mixed with loading buffer (sodium dodecyl sulfate [SDS], bromophenol blue, Tris Base, glycerol, mercaptoethanol, and milliQ water) and boiled at 95°C for 5 min. After short centrifugation, equal amounts (10 $\mu$g protein) were loaded on Criterion$^\text{TM}$ XT precast gels (Bio-Rad). After SDS-polyacrylamide gel electrophoresis, gel content was transferred to Immobilon-FL membranes (Merck Millipore, Billerica, USA) by tank blotting. Membranes were blocked for 1 h in Odyssey blocking buffer (LI-COR, Westburg, Leusden, The Netherlands) at room temperature. Thereafter, membranes were incubated overnight at 4°C in Odyssey blocking buffer containing the primary antibodies anti-myosin (MF-20, DSHB, Iowa City, IA, 1:20,000) and anti-actin (Sigma-Aldrich, Zwijndrecht, the Netherlands 1:500) and 0.1% Tween-20. After washing with ice cold TBS-T, membranes were incubated with IRDye 800CW goat anti-rabbit and 680CW goat anti-mouse antibodies in Odyssey blocking buffer with 0.1% Tween-20 for 1 h at room temperature. After washing with TBS-T, 2-color fluorescent bands were visualized by a 2-channel laser system, bands were identified by their molecular weight (myosin 200 kDa, actin 42 kDa), and were quantified (Odyssey IR Imager®; LI-COR Biosciences, Bad Homburg, Germany). Equal loading of the protein to the gel was ensured by Coomassie blue staining.

**Power Calculation and Statistical Analysis.** A power calculation was not performed for this study, because no data on grip strength in this animal model were available to support a power calculation.

Depending on the distribution of the data, means with standard deviation (±SD), medians with interquartile range (IQR), or range and proportions with percentages and total numbers are presented. The Welch t-test was used for assessment of differences between normally distributed variables, and the Wilcoxon rank-sum test was used for differences between nonnormally distributed continuous variables.

Differences in grip strength over time between the groups were studied with linear mixed effects models to account for repeated measures. Separate models for young and old mice were made. As fixed effects, E. coli (no/yes) and antibiotics (no/yes) were entered into the model. A mouse identification number was included as a random effect. Round (1/2) added as a fixed effect did not improve model fit and was therefore not included in the final model.

We did 2 subgroup analyses of more severely ill mice: (a) mice with bacterial outgrowth in blood and (b) mice with severe weight loss (>10% maximal weight loss).

Statistical significance was defined as $P < 0.05$. Analyses were done using R (version: 3.02; R Foundation for Statistical Computing, Vienna, Austria).

**RESULTS**

**Body Weight and Bacterial Outgrowth.** In young mice, the maximum weight loss in E+$A$ mice
compared with baseline body weight was 10.4% (median, IQR 9.7–11.7) versus 2.5% in C1A mice (median, IQR 1.9–2.9) and 2.1% in C mice (median, IQR 1.5–3.4) (P < 0.001). In old mice, the maximum weight loss in E1A mice was 7.2% (median, IQR 5.3–8.5) versus 1.0% in C1A mice (median, IQR 0.0–1.4) and 0% in C mice (median, IQR 0.7–1.1) (P < 0.001).

One mouse in the E1A group died due to severe illness before the end of the experiment.

In the 30 young mice of the E1A group, 22 (73%) showed bacterial outgrowth in spleen, 8 (27%) in liver, and none in blood. In 29 old mice, 25 (86%) showed bacterial outgrowth in spleen, 13 (45%) in liver, and 8 (28%) in blood.

**Grip Strength.** At baseline, mean normalized grip strength was lower in old mice (3.5 g/g body weight, SD 0.4) compared with young mice (4.4 g/g body weight, SD 0.5; P < 0.01) (Fig. 1). A decrease in grip strength over time was seen in all groups. *E. coli* infection was not associated with a decrease in grip strength (Table 2). In the model for young mice, antibiotic administration was associated with a minor increase in grip strength (Table 2).

Subgroup analysis of 8 old mice with bacterial outgrowth in blood showed similar results compared with the whole group analysis of old mice.

Also, subgroup analysis of animals with a maximum weight loss ≥ 10% (16 young and 5 old mice) showed similar results.

**DISCUSSION**

In this *E. coli* septic peritonitis model, there was no difference in grip strength decline between mice with *E. coli* infection and the control groups, in young as well as old mice.

Both infection and control groups showed a decline in grip strength between 24 and 48 h. This might be explained by learning bias, because frequent testing has been reported to lead to a loss of interest in holding the grip.17,18 The unwillingness of the mice also requires multiple trials per animal per time point to generate reliable data.17 Only 2 other studies used *in vivo* strength measurements in awake animals to investigate an ICU-AW model.19,20 Files et al. used the same test as we did and investigated grip strength in mice after intratracheal lipopolysaccharide administration.19 They found a decline in grip strength within 2 days, which remained stable thereafter until 9 days. However, the sham group also showed a temporary decline. Another method of strength assessment was used in a cecal ligation and puncture (CLP) model in rats. Half of the rats in the CLP group were unable to stand on a rotated screen at 7, 14, and 21 days after CLP, while sham rats did not show weakness.20 The use of CLP as a sepsis model is under debate; low consistency is a major

Table 2. Mixed effects models for the effect of *E. coli* and antibiotics on normalized grip strength.

|                  | Predicted effect on grip strength (g/g) | 95% Confidence interval | P-Value |
|------------------|-----------------------------------------|-------------------------|---------|
| Model young mice | E. coli                                | −0.13                   | −0.44–0.19 | 0.42 |
|                  | Antibiotics                            | 0.42                    | 0.04–0.79  | 0.03 |
| Model old mice   | E. coli                                | −0.06                   | −0.32–0.20 | 0.64 |
|                  | Antibiotics                            | −0.12                   | −0.43–0.19 | 0.44 |

**Western Blot Myosin/Actin Ratio.** In young mice no differences in the myosin/actin ratio were seen between E+A and C+A groups (Figs. 2 and 3). In old mice killed at 48 h, the myosin/actin ratio was lower in the E+A group compared with the C + A group (P = 0.01). An opposite effect was found in old mice killed at 72 h with a higher myosin/actin ratio in the E+A group compared with the C + A group (P = 0.01).

**FIGURE 2.** Western blot of myosin (upper bands) and actin (lower bands). C+A = control and antibiotics, E+A = *E. coli* and antibiotics.
problem, because outcome after CLP is associated with the technical procedures.  

**Age Dependent Grip Strength.** In healthy human and mouse models, grip strength decreases with increasing age. This is the first animal study to assess age-dependent grip strength in a sepsis model. Because grip strength is used as a marker of frailty, and age is a risk factor for developing ICU-AW, one would expect that older mice are more susceptible to a decline in grip strength than younger mice. In our model, grip strength at baseline was lower in old mice, as expected; however, the decline in grip strength did not seem to be different between young and old mice.

**Myosin/Actin Ratio.** In patients with ICU-AW, a decreased myosin/actin ratio is seen within 5 days after ICU-admission. In old mice, there was a difference in the myosin/actin ratio between the C+A and the E+A group. At 48 h after *E. coli* administration the myosin/actin ratio was lower in the E+A group, whereas it was higher at 72 h. The low myosin/actin ratio at 48 h might reflect a subclinical ICU-AW, because grip strength was not lower in the E+A group. This is an important finding pointing to the need to interpret *in vitro* findings together with functional measurements to ascertain their relevance. An overshoot of compensatory production of myosin might explain the
higher ratio at 72 h, although in humans persistent myosin loss has been seen until day 15 after ICU admission.\textsuperscript{23} We did not assess this further in our model.

**Suitability of this Model to Study ICU-AW.** No difference in grip strength decline was found between mice with *E. coli* infection and the control groups, irrespective of age. Therefore, the *E. coli* mouse model, in its current form, seems to be unsuitable to study ICU-AW.

Several factors may be important. First, the illness in this model may not be sufficiently severe and prolonged, so that the threshold to develop ICU-AW was not reached in young or old mice. Previous experiments with this model without antibiotic administration showed full-blown sepsis at 15 h after induction and a high mortality of 75 to 100% within 48 h.\textsuperscript{12} In our experiment, early antibiotic administration may have prevented prolonged illness. Although we found significant weight loss, indicating illness, none of the young and only 28% of old mice showed bacterial outgrowth in blood. Subgroup analyses of mice with bacterial outgrowth in blood and mice with severe weight loss did not show different grip strength results.

Second, grip strength testing may not be the best method to assess *in vivo* muscle strength in awake mice, because frequent measurements seem to cause learning bias. Other methods to assess *in vivo* muscle strength such as the rotarod test or inverted screen test might be useful to detect changes in strength over time in awake mice.\textsuperscript{17} Finally, the duration of this model might be too short to detect weakness. In other animal models, it took several days before weakness was detectable.\textsuperscript{19,20} Even so, learning bias may hamper sequential strength measurements in longer duration models.

**Limitations of this Study.** We did not perform a power calculation, as this was an exploratory study on grip strength, which was never measured in this model before. However, the exploratory nature of this study allowed us to combine animal experiments and contributed to use of fewer animals. This experiment was primarily designed and powered to study microglia activation in sepsis.

Because of combining animal experiments we were not able to perform other *in vivo* strength measurements or electrophysiological studies.

**Recommendations for Future Research.** To study ICU-AW, an animal model with a decline in muscle strength, assessed with *in vivo* strength measurements in awake mice during sepsis, is needed, because a decline in muscle strength is the most important feature of ICU-AW in humans. A prolonged model, with prolonged and severe critical illness might be needed to induce *in vivo* ICU-AW in mice.

In conclusion, this *E. coli* septic peritonitis mouse model did not induce decreased grip strength. No effect of age on grip strength decline could be found in this model.

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