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Short communication

Establishment of an objective endpoint in mice model for caseous lymphadenitis vaccine trials

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ABSTRACT

For a long time, the scientific community has described the need for a continued update in practices that ensure the welfare of animals undergoing experimentation. In addition to approaches on principles of care and use of animals, there is a more current emerging concern: defining an appropriate end point in experiments that use animals for research, teaching and testing. The term "endpoint" is defined as the point at which an experimental animal's pain and/or distress is terminated, minimized, or reduced humanely. In the present study, we established an endpoint in Balb/C mice for caseous lymphadenitis vaccine trials, which can be considered as a highly important parameter since several studies are being developed to control the disease efficiently. Mice were monitored daily until the 30th day after infection with pathogenic strain of *C. pseudotuberculosis* MIC–6 using the most relevant parameters for the appearance of clinical signs of caseous lymphadenitis (CLA), such as abscesses, lethargy, and loss of weight and hair. The endpoint was found to be a weight loss of 0.2167 g after five days or 10% weight loss in less than five days. In conclusion, the findings reported here will help improve animal's well-being during vaccine trials for CLA and consequently represent significant contribution to animal's welfare.

1. Introduction

In the past, several studies involving animal models for different diseases had the animal's death as the endpoint of the experiment, but this requirement is now questioned (CCAC, 1998). The term "endpoint" is defined as the point at which an experimental animal's pain and/or distress is terminated, minimized, or reduced humanely (CCAC, 1998). According to the approved method (CCAC, 1991), animals must be euthanized after recognition of a status where it exhibits a high level of pain or distress without any chance of re-establishing the health parameters.

To date, no data regarding endpoint for caseous lymphadenitis (CLA) are available. In addition, clinical trials performed for vaccine development against CLA involve administering the animals a high dose of bacteria (10^4-10^6 CFU) during challenge assay that generally results in septicemia and death, thereby causing great suffering to animals (Costa et al., 2011; Ribeiro et al., 2014; Brum et al., 2017).

CLA is an infectious-contagious bacterial disease characterized by the presence of abscesses in the peripheral lymph nodes (superficial form) and/or internal organs (visceral form) and it affects mainly small ruminants (sheep and goats) around the world, causing significant economic losses (Williamson, 2001; Dorella et al., 2006). The prevalence of CLA is high in many regions worldwide, including South America (Seyffert et al., 2010; Droppa-Almeida et al., 2016). In southeast of Brazil, a study conducted in goats has reported a seroprevalence of 78% (Seyffert et al., 2010), while the prevalence in sheep was estimated as 75.8% (Guimarães et al., 2011). Additionally, epidemiological studies have estimated that the clinical prevalence in Brazilian herds exceeds 30% (de Sá Guimarães et al., 2011).

Several studies have been conducted aiming to develop preventive measures to control the disease (Silva et al., 2014, 2018; Droppa-Almeida et al., 2016; Brum et al., 2017), using Balb/c mice as an experimental model. Despite many advances made in the research against *Corynebacterium pseudotuberculosis* (etiologic agent of CLA), a vaccine providing 100% protection rate is still not available (Bastos et al., 2012). Therefore, more rigorous and rapidly evolving studies are required, which, in turn, would require more animals for experimental testing. In this context, the present study aimed at establishing an

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appropriate endpoint in experiments using mice Balb/C in research on the CLA vaccines.

2. Materials and methods

2.1. Animals and ethics statement

Twenty clinically healthy Balb/c female mice (6–8 week old and approximately 30 g), susceptible to *C. pseudotuberculosis* infection were used in the study. The animals were obtained from the Central Animal Facility of the Federal University of Pelotas, where all experiments were conducted, and the study was approved by the Ethics Commission on Animal Experimentation of the Pelotas Federal University (number 2442). All mice used in the study were humanely housed, being allocated in polycarbonate cage of solid bottom counting with appropriate bedding material, water, and food *ad libitum*. The presence of a maximum of 10 animals per cage was determined to ensure better comfort level for the animals.

2.2. Groups

After an acclimatization period, a total of 20 mice were allocated equally into two groups: not challenged (G1) and challenged (G2). Both groups were inoculated subcutaneously with 0.9% saline formulation in two doses with a 21-day interval to mimic the vaccination protocol (Brum et al., 2017; Silva et al., 2018).

2.3. LD_{50} determination and challenge

As the first step for our study, we used the pathogenic strain of *C. pseudotuberculosis* MIC–6 (assembly: GCA_002009415.1) to determine the minimum dose to induce a lethal infection in 50% of the animals (LD_{50}), which was subsequently used in determining the challenge dose concentration. The LD_{50} was determined by inoculating serial doses of bacteria according to the previously described protocol (Simmons et al., 1997). Thus, six groups with 4 animals each were infected intraperitoneally with serial doses ranging from 10^1 to 10^6 colony forming units (CFU). The LD_{50} was defined as the amount of CFU leading to the death of 50% animals in a group (Reed and Muench, 1938). The animals from G2 were challenged with twice the value of LD_{50} obtained through the above mentioned experiment.

2.4. Monitoring assessments

The animals of both groups were observed daily at the same time in the morning. The animals were monitored for seven days before the challenge assay until thirty days after the assay, using the most relevant parameters for the appearance of clinical signs of CLA, such as abscesses, lethargy, and loss of weight and hair following the appropriate observations described by Morton and Griffiths, (1985). A table was formulated that listed symptoms of all animals separately over the days to compare the groups (data not shown). All observations were validated by two independent experiments, performed at different times, in order to attest and ensure reproducibility.

2.5. Statistical analysis

Student's *t*-test was used to determine statistically significant differences between the initial and final average weights obtained for animals in G1 and G2, and between the percentages of weight change. The loss or gain in weight inside the groups was determined using the linear regression model. All statistical analyses were performed using the GraphPad Prism version 5.0 for Windows (GraphPad Software).

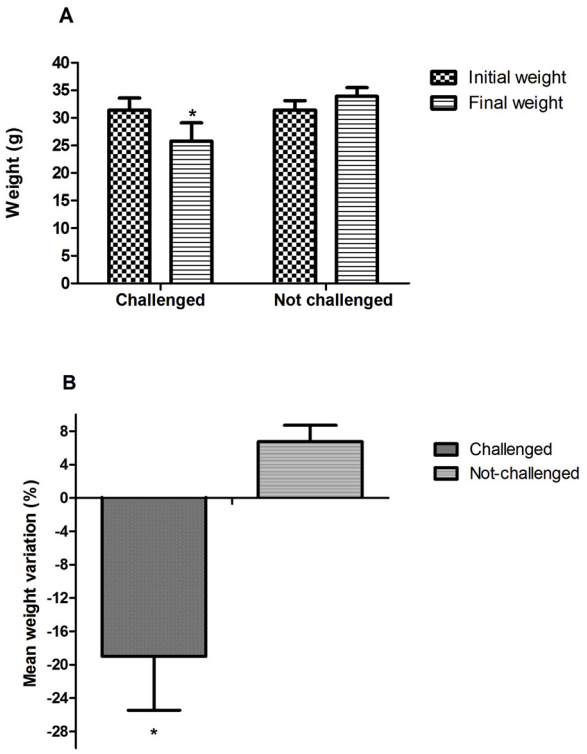

Fig. 1. The comparison between the weight of the animals during the initial and final days of the experiment. (A): Mean weight variation per group. (B): Mean percentage of weight variation per group. Negative values refer to the percentage of weight loss, while the weight gain is represented by positive values. All animals were weighed daily at the same time up to 30 days after the challenge. In the group with challenged animals, the weight of the day before the death was considered as final weight. Significant differences between the mean weights in the different groups were calculated employing Student's *t*-test. *group significantly different ($p < 0.05$).

3. Results and discussion

To the best of our knowledge, the present study is the first to determine a humanized endpoint criterion for an experimental model for CLA in vaccine survival trials.

In our study, the LD_{50} was observed as being 10^4 CFU, and the animals from G2 were challenged with a dose containing 2×10^4 CFU of *C. pseudotuberculosis* MIC–6 strain. From the daily monitoring of mice for 37 days (7 days before and 30 days after the challenge), we evaluated a combination of parameters (weight change analysis, abscess formation, lethargy and loss of hair) to establish a reliable endpoint. The analysis of weight loss was the main indicator of inability to recover from the infection in mice. It was thus a potential option to determine human intervention since challenged animals (G2) reported reduced weight whereas non-challenged animals reported a gain in weight (Fig. 1A). After infection with MIC–6 in G2, mice lost weight, became weak, and a mortality rate of 100% was reported in this study. The assessment of body weight has already demonstrated predictive qualities related to overall loss of body condition during experimental manipulations (Toth et al., 1995). In G2, we observed an average negative variation of 19% (Fig. 1B) in animal weight, whereas G1 showed an average weight gain of 6.75% (Fig. 1B). In our experiments, the abscess formation and the lethargic activity also contributed to general health assessments, giving more information about the stage of infection in G2. Two animals in G2 established a state of lethargy a few days before death; superficial abscesses were observed in one animal 15 days after the challenge. However, as demonstrated by Leal et al. (2018), MIC–6 strain is considered to have the ability to kill mice and to disseminate the infection, leading to acute shock. So, there is no time for

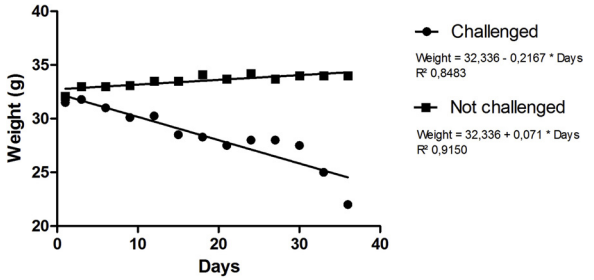

Fig. 2. Linear regression of the animal weight throughout the days in the challenged and not challenged groups. Each point or square corresponds to a three-day observation of weight. The lines demonstrate an $R^2 = 1$. The challenged group showed a weight loss of 0.2167 g (R^2 0.8483), and the not challenged group showed a weight gain of 0.071 g (R^2 0.9150) showing the opposite coefficient profile.

some infection characteristic symptoms manifest and thus, lethargy and abscess formation are not reliable parameters for establishing an endpoint. The loss of hair was not considered suitable as an endpoint, since no significant differences related to this characteristic were observed between G1 and G2.

The linear regression analysis reported a weight loss of 0.2167 g (R^2: 0.8483) daily after challenge in G2 over the experimental course. On the other hand, mice in G1 showed a weight gain of 0.071 g (R^2: 0.9150) daily. Consequently, the data (from G1 and G2) did not intersect owing to the opposite coefficient profile (Fig. 2). Therefore, we recommend the continued weight loss of 0.2167 g daily in 5 consecutive days (period sufficient and necessary to differentiate losses that would naturally occur due to a lower intake of food or other external causes of losses caused by the infection) as an endpoint, or an observation of a weight loss of 10% in up to five days (in cases of sudden weight loss and abrupt death). Both criteria indicated toward no chance of recovery from the infection and animal progressing to rather death. Thus, we determined the exact time of a human intervention. This parameter allowed 100% specificity for successfully reaching the planned experimental endpoint.

Appropriate endpoints have already been established for other diseases and their results are similar to those obtained in our study. In leptospirosis, the decrease in body weight is the earliest clinical sign to be observed; therefore, a criterion of 10% weight loss prevents the occurrence of spontaneous death (Coutinho et al., 2011, 2014). Herpes simplex virus (HSV) model accepts a combination of criteria including body temperature (less than 34.5 °C) and weight loss (more than 0.05 g daily) to be sufficient for removal of animals from the study (Hankenson et al., 2013).

The endpoint obtained in our study is greatly significant and its application has the potential to improve animal's welfare in future studies of CLA, by making possible to detect signs of progressive deterioration in the animal's physical conditions that would eventually lead to its death (Acred et al., 1994). This endpoint allows to guarantee that the suffering of the animals used in the experiment is alleviated or minimized without influencing the final outcome of the study. In addition, the present study gives support to the efforts to continuously improve animal research practices, which we believe is a responsibility of all people involved in a research project.

In conclusion, these findings would significantly contribute to improving animal welfare during future vaccine trials for CLA until an ideal vaccine is obtained. Results obtained here are efficient, easily applicable, and allow researchers to better control their experiments, while protecting animals from suffering, by closely monitoring animals' weight. Finally, the data presented here has the potential to be applied in many research groups that work with CLA and can help with the standardization of animal's handling, monitoring, and welfare in this field of research.

Conflict of interest statement

The authors declared no potential conflicts of interest with respect to the research, authorship, or publication of this article.

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