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Optimisation of bronchoalveolar lavage technique for isolating alveolar macrophages in mice

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Bronchoalveolar lavage (BAL) is a widely used technique to collect immune cells from the lungs, with alveolar macrophages (AMs) being the most prevalent cells in BAL fluid. AMs are vital for maintaining lung homeostasis and providing immune defence against airborne pathogens. However, in murine models, BAL procedures usually yield low numbers of AMs, thus limiting experimental design, especially when high cell counts are needed.

Objective — to optimise BAL techniques in mice to maximise AM recovery.

Materials and methods. Young and older BALB/c mice were used in the study. Bronchoalveolar lavage was performed following the method of Luckow and Lehmann (2021) with modifications. Statistical analysis was done using the Mann-Whitney U test, with a significance level set at $p < 0.05$.

Results. Female BALB/c mice of different ages were chosen due to the frequency of their use as models of pulmonary diseases. A simplified method described by Luckow and Lehmann (2021), which avoids tracheotomy by using peroral cannula insertion, was employed. The protocol was modified by securing the cannula with a ligature to prevent BAL fluid leakage in older mice. To reduce mechanical stress on alveoli, a buffer volume of 0.6 mL was used, and the study compared two buffer variants: one at room temperature without EDTA, and another heated to 37°C with EDTA. The pre-heated buffer with EDTA significantly increased BAL cell yields in all mice groups, confirming the importance of these optimisations for higher cell recovery.

Conclusions. Our modified bronchoalveolar lavage protocol includes securing the trachea with a ligature to prevent BAL fluid leakage, reducing lavage volume to 0.6 mL to minimise lung damage, and using a 37°C solution with EDTA for improved AM recovery rates. Further studies are needed to explore the significance of other buffer components for BAL protocol optimisation, the possible age-related differences in AM isolation in male BALB/c mice, and the strain-specific features of the BAL technique.

Keywords
bronchoalveolar lavage, alveolar macrophages, aging.

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Bronchoalveolar lavage (BAL) is used to isolate immune cells and soluble substances from the lungs [4]. Under normal conditions, alveolar macrophages (AMs) are the predominant cell type collected in BAL fluid [27]. In addition to homeostatic functions, i.e., removal of lung surfactant and cellular debris, this population of macrophages is important for the protection of the host from airborne pathogens. They can recognise pathogens and destroy them through phagocytosis, as well as provide the necessary stimuli to engage adaptive immunity in a fight against infection [16]. In light of the aforementioned, research

on AMs is vital for improving host resistance to infections spread by aerosol transmission, including emerging virus variants with epidemic and pandemic potential, such as SARS-CoV-2 [7], as well as research on the role these cells play in the pathogenesis of non-infectious lung diseases [19].

Mice are commonly used in laboratories because they are easy to handle and care for, share a high degree of similarity with humans, and reproduce quickly [2]. Animal models, including murine ones, are the pillar of pulmonary research since they reproduce the numerous complex physiological processes, multiorgan interplay, and disease phenotypes. Murine models are widely used for preclinical experiments concerning emerging infectious diseases, asthma, chronic obstructive pulmonary diseases, lung cancer, etc. [25]. Pulmonary macrophages, including AMs, are the principal object in such models. In these experiments, mice's age is one of the crucially important factors, on which depends the translational potential of the experimental study [9]. Animal sex also plays a crucial role, especially in studies that focus on immune reactivity and consider sex-dependent characteristics of immunity [6]. All of these factors require the incorporation of cell isolation techniques in the study design, considering animal age and sex characteristics.

BAL usually yields a relatively low number of AMs per mouse, often less than $1 \cdot 10^5$ [15, 28], which may significantly limit experimental design. Using more mice to address this issue contradicts the «4Rs» principle of animal research, which mandates limiting the animal count to the bare minimum required for dependable data [11].

Objective — to optimise the BAL technique in mice to maximise the collection of AMs.

Materials and methods

Ethical statement. Young (6—12 weeks old) BALB/c mice of both sexes and older (8—10 months old) female BALB/c mice were used in experiments. Animals were euthanised by injecting an overdose of anesthetic (sodium thiopental 200 mg/kg) into the peritoneal cavity. The animal procedures received approval from the Bioethics Committee of the Educational and Scientific Centre «Institute of Biology and Medicine» at Taras Shevchenko National University of Kyiv.

Bronchoalveolar lavage. The method recently described by Luckow and Lehmann (2021) was used with modifications [14]. Briefly, the skin on the ventral side of the neck is disinfected using ethanol, and an incision is made with scissors to reveal the salivary glands that lie over the trachea. The salivary glands are carefully removed with forceps to expose the trachea. A 1 ml syringe filled with buffer is attached to a rigid straight 23G olive tip cannula (J0156A; Jorgensen Laboratories, LLC, USA). The olive on the tip is polished and smooth, free from sharp edges. The syringe with the cannula is then inserted through the mouth directly into the trachea, ensuring that the esophagus is not entered. Once the cannula is correctly positioned, it is tied by ligature placed posterior to the olive tip. Then, 0.6 ml of lavaging solution (saline warmed to 37 °C and supplemented with 2 mM EDTA) is gradually introduced into the lungs and then slowly aspirated. The fluid is collected in a tube after detaching the syringe from the cannula. BAL was then repeated 3 times, with the cannula staying inside the trachea.

Statistical analysis. Mann-Whitney U test was used for comparisons between groups. The significance level was set at $p < 0.05$.

Results and discussion

The BALB/c strain was chosen for these experiments due to its common use in preclinical models of pulmonary diseases [25]. Additionally, we have used female mice of different ages considering their frequent use in models of chronic obstructive pulmonary disease and asthma — diseases with sex-dependent prevalence [10, 18].

The majority of the described BAL methods involve performing a tracheotomy with the subsequent insertion of a cannula into the trachea [4]. The cannula is also fixed with a surgical knot to prevent it from sliding out of the trachea and reduce leakage of BAL fluid. Due to the small size of mice, this procedure is quite complex and may require a lot of trained personnel when high numbers of AMs are needed for study. Therefore, we chose a simplified protocol recently published by Luckow and Lehmann (2021), which is based on the peroral insertion of a cannula and thus does not require tracheotomy [14].

According to Luckow and Lehmann (2021), the olive tip of the cannula has a larger diameter than the internal lumen of the murine trachea. Therefore, the tracheal muscles ensure consistent fixation of the cannula, thus preventing the leakage of BAL fluid. The authors applied their technique on adult animals with weights ranging from 15 to 35 g [14]. Although suitable for the majority of experiments on mice, this method may fail in ageing studies. Since mice grow bigger with age, the diameter of their tracheal lumen may proportionally increase [5, 13]. Additionally, the contractile properties of smooth muscle cells, including tracheal muscles, can deteriorate with ageing [1]. This may explain frequent BAL fluid leaking, which

was observed in our pilot studies on older mice. Hence, we modified this method by placing a ligature around the trachea posterior to the olive tip, thus firmly securing the inserted cannula (Fig. 1).

One of the important parameters of the BAL procedure is the volume of the instilled buffer. 1 mL is frequently used as a fixed one-time volume of the lavage buffer that is repeatedly injected into the lungs. According to Lai and Chou, total lung capacity and functional residual capacity in mice are 1.05 ± 0.04 and 0.25 ± 0.01 mL, respectively [12]. Consequently, the infusion of 1.0 mL buffer in one go during BAL fluid collection could impose mechanical stress on the alveoli, potentially causing harm to macrophages [17, 24]. These assumptions were confirmed by Sasaki et al. They compared 3 different fixed volumes of injection buffer (0.55 mL, 0.75 mL, and 1 mL, instilled 10 times each) and found that an infusion of 1mL leads to the highest percentages of CD11b-CD11c-SiglecF-cells (presumably alveolar epithelial cells) in BAL fluid compared to two other studied volumes. Moreover, detrimental effects were observed in AMs isolated with 1mL of injection buffer, including reduced cell viability during culture and impaired functionality, particularly in response to stimulation with fine particles. Notably, the authors did not register a significant influence of lavaging fluid volume on recovery efficiency [22]. In light of the aforementioned factors, a fixed injection volume of 0.6 mL was chosen for our studies.

EDTA is a chelating agent that is often used in the laboratory to detach adherent cells. It chelates calcium and magnesium, leading to cadherin misfolding and disruption of lateral cell junctions [21]. It is sometimes added into injection buffer to facilitate detachment of AMs from alveoli [8, 20], but other researchers do not use it [23]. The temperature of the lavaging fluid also varies in different reports: some authors use pre-cooled buffer (4 °C) [15], while others heat it to 37 °C [8, 26]. Busch et al. have found that both the addition of EDTA and pre-warming of lavage buffer to 37 °C yield more AMs than cold buffer without EDTA [3].

In our study, 2 variants of lavage buffer were compared:

1) Room temperature without EDTA (buffer No 1);

2) heated to 37 °C and supplemented with EDTA (buffer No 2).

Buffer No 1 yielded a maximum of $3 \cdot 10^5$ cells in all studied groups of mice, while buffer No 2 increased the numbers up to $5 \cdot 10^5$ cells in young animals of both sexes and more than $10 \cdot 10^5$ cells in old female BALB/c mice (Fig. 2). Overall, our observations show that using a pre-heated injection buffer containing EDTA resulted in significantly higher BAL cell numbers in BALB/c mice regardless of age and sex. Our results confirm the findings of Busch et al., which suggest that pre-warming and EDTA supplementation are important optimisations that lead to significantly higher BAL cell numbers in mice.

Conclusions

Our BAL protocol variant features several important developments:

1) Securing the trachea with a ligature prevented BAL fluid leakage in older mice, thereby expanding Luckow and Lehmann's technique (2021) to bigger and older animals.

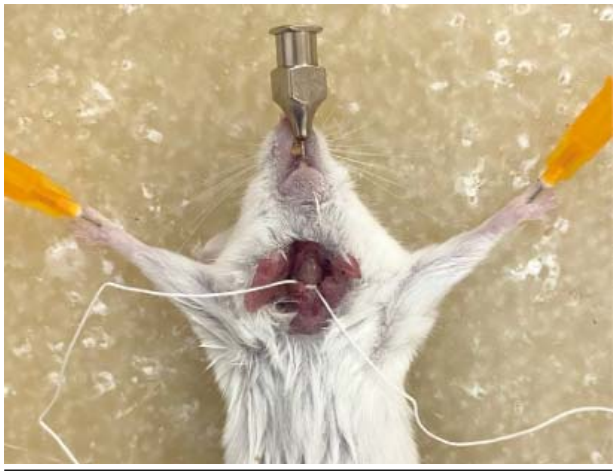
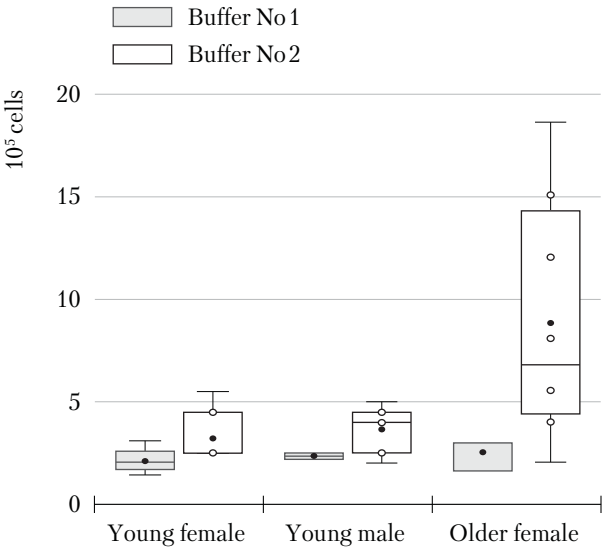


Figure 1. **Ligature securing cannula inside the trachea**



The difference between buffer No 1 and buffer No 2 is statistically significant for all three groups ($p < 0.05$).

Figure 2. **Comparison of cell numbers yielded by buffer No 1 and buffer No 2 in young female, male, and older female BALB/c mice**

2) The reduction of the one-time lavage volume to 0.6 mL minimised mechanical strain on lung alveoli and, thus, prevented AM damage.

3) The use of a pre-heated 37 °C lavage solution containing EDTA significantly enhanced AM recovery rates, particularly in older mice.

More studies are warranted to explore the role of other potential lavage buffer components, such as fetal bovine serum, as well as to further adjust its volume, EDTA concentration, and other parameters. Assessing age-related features in AM isolation using BAL is needed in male BALB/c animals. Moreover, one cannot exclude strain-specific features of the BAL technique.

Declaration of interests

The authors declare no competing interests.

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Authors contributions

Conception and design, critical review: L. Skivka; data collection: R. Dovhyi, M. Rudyk, A. Dvukhriadkina, K. Ostrovska; data analysis and interpretation: R. Dovhyi, T. Serhiichuk, Y. Yumyna, N. Senchylo; drafting the manuscript: R. Dovhyi.

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Оптимізація методики бронхоальвеолярного лаважу для виділення альвеолярних макрофагів у мишей

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Бронхоальвеолярний лаваж (БАЛ) є широко використовуваною методикою для виділення імунних клітин легень, при цьому альвеолярні макрофаги (АМ) є найбільш численними клітинами БАЛ. АМ відіграють ключову роль у підтримці гомеостазу легень та імунному захисті від патогенів, що передаються повітряним шляхом. Однак при проведенні БАЛ на мишах зазвичай вдається отримати відносно малу кількість АМ, що значно обмежує можливості дизайну дослідження, особливо коли потрібно отримати велику кількість клітин.

Мета — оптимізувати методику БАЛ у мишей для отримання максимальної кількості АМ.

Матеріали та методи. У дослідженні використовувалися молоді та старші миші лінії BALB/c. БАЛ отримували за методикою Luckow and Lehmann (2021) з модифікаціями. Статистичний аналіз виконували за допомогою U-тесту Манна — Уїтні, при рівні значущості $p < 0,05$.

Результати. Для досліджень було обрано самиць мишей лінії BALB/c різного віку у зв'язку з частим використанням таких тварин для моделювання захворювань легень. Використовувався спрощений метод, описаний Luckow and Lehmann (2021), який уникає застосування трахеотомії завдяки пероральному введенню канюлі. Протокол було модифіковано шляхом закріплення канюлі лігатурою для запобігання витоку рідини БАЛ у старших мишей. Для зменшення механічного навантаження на альвеоли використовували об'єм буфера 0,6 мл. В даному дослідженні порівнювали два варіанти буфера: один кімнатної температури без ЕДТА, а інший — підігрітий до 37 °C з ЕДТА. Застосування попередньо підігрітого буфера з ЕДТА значно збільшувало кількість клітин БАЛ у всіх групах мишей, що підтверджує важливість такої оптимізації протоколу для збільшення кількості АМ.

Висновки. Наш модифікований протокол бронхоальвеолярного лаважу включає закріплення трахеї лігатурою для запобігання витоку рідини БАЛ, зменшення об'єму лаважу до 0,6 мл для мінімізації пошкодження легень, і використання розчину, підігрітого до 37 °C з ЕДТА для збільшення кількості АМ. Потрібні подальші дослідження з метою вивчення значення інших компонентів буфера для оптимізації протоколу БАЛ, можливих вікових відмінностей у виділенні АМ у самців мишей лінії BALB/c, а також особливостей застосування техніки БАЛ на мишах різних ліній.

Ключові слова: бронхоальвеолярний лаваж, альвеолярні макрофаги, старіння.

FOR CITATION

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