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The Effect of Time and Holding Media on Cell Derivation of Balb/C Mouse at Room Temperature

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ABSTRACT

Efficient cell derivation from post-mortem tissues is critical for biomedical research, yet its success is often compromised by delayed processing and inadequate preservation. This study aimed to assess the impact of post-mortem time intervals and the use of holding media on the success rate of cell derivation from Balb/C mice tissues at room temperature. Tissue samples were divided into four groups based on time (0 hours, 6 hours, 12 hours, and 24 hours post-mortem) and whether they were retained in media or not. Then, the samples were processed by washing, mincing, neutralising, centrifuging at 1000 rpm for 5 minutes, and resuspending before plating in 12-well plates in triplicates. The data were analysed using RStudio. Tissues retained in media maintained viable cell morphology and a gradual decline in plating efficiency up to 24 hours (75%), whereas tissues without media showed loss of viability as early as 6 hours, with plating efficiency dropping to 8.33% and declining further over time. Statistical analysis revealed a p-value of 0.283 for tissues retained in media, indicating no significant difference in viability over time, whereas tissues without media had a p-value <0.001, indicating a significant time-dependent decline. The time factor alone showed no significant impact on derivation success for tissues in media, maintaining tissue hydration prior to derivation is crucial for ensuring higher viability and successful outcomes. It is hoped that this study could serve as a foundation for

future research, further advancing efforts and activities related to planetary health.

INTRODUCTION

The time of death is a crucial factor in cell derivation, as it significantly impacts cell viability and quality. After an organism's death, cells rapidly deteriorate due to the depletion of oxygen and nutrients, triggering enzymatic and biochemical degradation (Lee & Overholtzer, 2019). Postmortem changes, which begin at the cellular level and progress over time, involve complex biological processes influenced by numerous factors (Almulhim & Menezes, 2025). A primary cell line is a culture of cells directly isolated from an organism's tissues or organs, commonly used in laboratory research to investigate cellular biology, physiology, and disease mechanisms (Piwocka et al., 2024). During the process of cell derivation of high-quality cells, tissue samples should be processed immediately, although this is often challenging due to the resource demands of cell isolation and expansion. (Deng et. al., 2024). The earlier the tissues has been derived, the greater the potential of preserving their integrity and functionality. However, the success of cell derivation is dependent on time of death and media condition. Understanding these factors is very essential for applications such as Frozen Zoo, a biobank that cryopreserves living cells, DNA, sperm, eggs, and embryos of endangered or extinct species. First appearing in scientific literature in 1996 to describe human population-based collections, the term biobank has since evolved in usage, now encompassing a broad range of definitions across reports, guidelines, and regulatory documents (Hewitt & Watson, 2013). Preserving viable samples in a biobank is not just ensuring that valuable genetic material is not lost but also provides the opportunity for future technological advancements to make use of these resources (Bolton et. al., 2022).

In wildlife conservation, establishing optimal conditions for post-mortem cell collection is essential to minimise resource wastage and improve the efficiency of biobanking efforts, particularly when sourcing samples from roadkill or abandoned carcasses. Tissue samples collected from roadkill wildlife offer valuable genetic data for understanding biodiversity, species identification, and pathogen monitoring, while enabling the creation of biobanks without additional impact on wildlife populations. (Coba-Males, et. al., 2023). The viability of cells derived from such sources is highly dependent on factors such as the time elapsed post-mortem, temperature of storage, and the presence or absence of preservation media. Without a clear understanding of how time affects successful cell derivation, researchers risk low success rates and the inefficient use of valuable biological materials, which are often rare and irreplaceable in the context of endangered species. Moreover, scientific experiments whether conducted in wet or dry labs are inherently resource-intensive and often contribute to environmental issues. As highlighted by Freese et al. (2024), these experiments frequently produce substantial plastic waste and hazardous chemical byproducts due to the reliance on single-use consumables and energy-demanding protocols. Consequently, refining collection and storage protocols, including determining whether tissues should be transported in preservation media or kept dry, is vital for enhancing cell viability. Such improvements not only support the success of conservation programs but also align with sustainable scientific practices by reducing unnecessary waste and maximising the use of every collected specimen.

To address these challenges, the present study was designed to evaluate the impact of post-mortem interval and holding media conditions on the success of primary cell derivation from BALB/c mice tissues maintained at room temperature. By systematically examining how delays in tissue processing and the choice of storage media affect the capability of the cells to be derived. Herein, this study aims to enhance practical protocols that improve the efficiency and reliability of primary cell derivation techniques. In addition, this study may provide valuable insights to wildlife conservation efforts, particularly in guiding the timely and effective collection of tissues from deceased animals. Furthermore, by identifying factors that maximise the preservation of viable cells, this research supports a long-term biobanking and species recovery programs, while promoting sustainable and responsible scientific practices.

MATERIALS AND METHODS

Ethical consideration

The ethical approval for conducting this study has been obtained from the institute and university ethical committees (IACUC 2023-015).

Complete media preparation

The conditioned culture media used for cell derivation comprised Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin and streptomycin).

Animal preparation

In this study, only one BALB/c mouse was used. The mouse was placed in a chamber and euthanised using the carbon dioxide (CO_2) inhalation technique. Death was confirmed by the absence of respiration. The mouse was dissected, and the muscle tissues were isolated from the bone. In this study, the tissues were classified into two groups: one group was kept in media, while the other was maintained without media during incubation at room temperature. The incubation periods were set at 0-, 6-, 12-, and 24-hours post-mortem prior to the cell derivation process. The flow of the study design is illustrated in Figure 1.

Cell derivation of the BALB/c mice

The protocol for the cell derivation has been adapted and modified from Abdul Razak et al. (2020). After the incubation periods (0, 6, 12, and 24 hours), the muscle tissues were washed with conditioned media. The tissues were then minced using a dissecting blade in a droplet of the proteolytic enzyme Accutase. The minced tissues, along with Accutase, were aspirated and transferred into a 15 ml conical tube. The tissues were left to incubate in Accutase for approximately 15 minutes. The conditioned media was added to the tube and set for centrifugation at 1000 rpm for 5 minutes. The supernatant was removed, and the pellet was resuspended in conditioned media. For each experimental condition, approximately 0.2 g of muscle tissue was seeded into a 12-well plate, with each condition plated in triplicate (four wells per replicate). The culture plates were incubated at 37°C with 5% carbon dioxide.

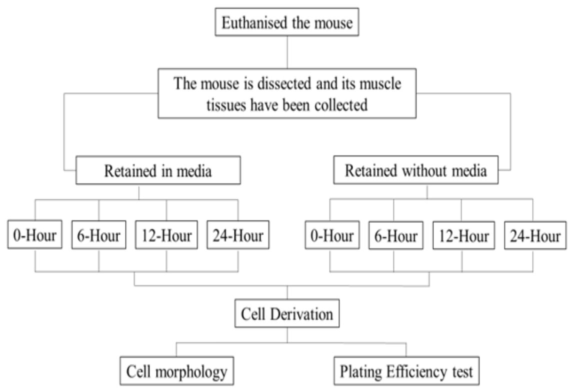

Figure 1: The flow chart of the study design

Morphology of the primary cells

The photographs of the cells have been capture using inverted microscope (EVOSTM XL Core, Invitrogen) post 72 hours of incubation. Images were taken at 10× magnification to observe cell morphology and attachment to the culture surface.

Plating efficiency calculation

In this study, the plating efficiency has been calculated to measure of the success of the cells to be derived based on two factors (time and holding media). It was employed as an initial qualitative screening approach to confirm successful cell outgrowth rather than as a definitive measure of cell viability or morphology. Plating efficiency was determined based on the presence of fibroblast-like cells attached to the surface of the culture wells after 72 hours of incubation, observed under a 10× magnification field using an inverted microscope. A well was considered successful for cell derivation if fibroblast-like cells were present and adhered to the culture surface. The calculation formula is as follows:

Plating efficiency % = (Number of with fibroblast-like / Total well seeded) × 100

Statistical analysis

Data analysis was performed using RStudio. Descriptive statistics, including means and standard deviations, were calculated to summarize the data. Pearson's Chi-square test was applied to evaluate the association between incubation time (Directly Derived, Post 6 Hours, Post 12 Hours, and Post 24 Hours), the presence or absence of holding media, and plating efficiency (successful vs. unsuccessful cell derivation). As all variables were categorical, normality testing was not applicable. The Chi-square test was selected to determine whether the distribution of plating efficiency outcomes differed significantly across the different incubation times and holding media conditions. A p-value of <0.05 was considered statistically significant.

RESULTS

Cell morphology post cell derivation

Figure 2 depicts the morphology of cells derived from the explant that was retained in holding media prior to cell derivation, observed at different time points (0 hour, 6 hours, 12 hours, and 24 hours). Fibroblast-like cells were successfully observed and derived at all time points after 72 hours of incubation in the carbon dioxide incubator. In contrast, successful cell derivation from tissue explants that were not retained in holding media was observed only up to the 6-hour time point, as illustrated in Figure 3. The cells exhibited spindle-shaped, elongated and either bipolar or multipolar centrally located nuclei.

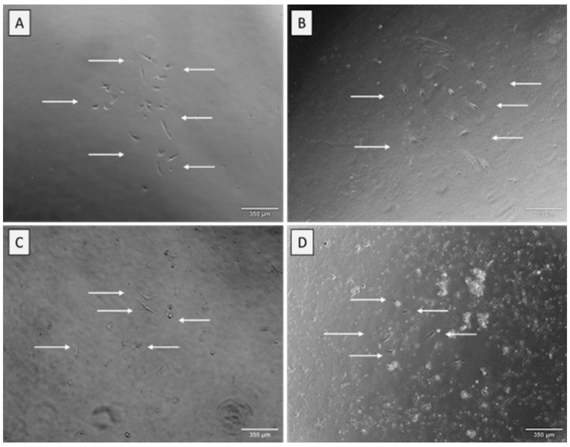

Figure 2: Morphology of cells derived from post-mortem tissues retained in conditioned media at room temperature across four time points: A) 0 hours, B) 6 hours, C) 12 hours, and D) 24 hours. White arrows indicate successfully derived cells. Images were captured at 10× magnification, with a scale bar representing 350 µm.

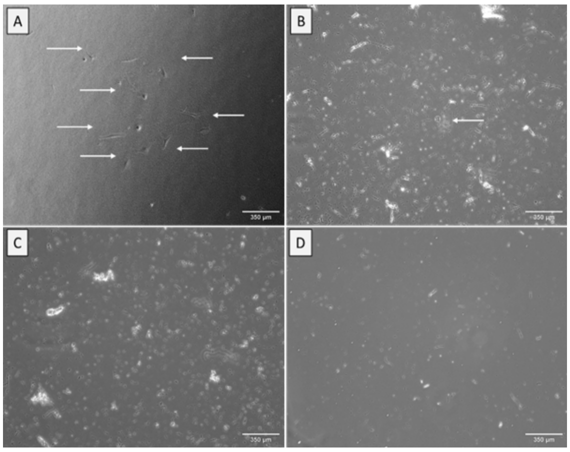

Figure 3: Morphology of cells derived from post-mortem tissues not retained in conditioned media at room temperature across four time points: A) 0 hours, B) 6 hours, C) 12 hours, and D) 24 hours. White arrows indicate successfully derived cells. Images were captured at 10× magnification, with a scale bar representing 350 µm.

Percentage of plating efficiency

This study assessed the success of cell derivation from muscle tissues collected from mouse that has been euthanised and incubate at varying time points postmortem, comparing tissues retained in holding media versus those left without media at room temperature. Table 1 shows the mean percentage values and their corresponding standard deviations (SD) for muscle tissue samples preserved in holding media (immersed) and those without media (non-immersed) across different time points (0 hour, 6 hours, 12 hours, and 24 hours). Figure 4 shows the plating efficiency of muscle tissues preserved in holding media compared to those without media. The muscle tissues preserved in holding media showed successful cell derivation for all different time points (0 hour, 6 hours, 12 hours, and 24 hours). However, it potrays a gradual declining pattern over time. In contrast, cell derivation from muscle tissues retained without any media was successful only at two time points, namely 0 hour and 6 hours. The pattern exhibits a rapid decline in cell derivation success, with no viable cell growth observed beyond 12 hours postmortem.

Table 1: The mean and standard deviation data.

Incubation Period before cell derivation	Immersed in media mean ± SD	Not Immersed in media mean ± SD
0-hour	100 ± 0	100 ± 0
6-hour	91.67 ± 14.434	8.33 ± 14.434
12-hour	83.33 ± 28.868	0 ± 0
24-hour	75 ± 43.301	0 ± 0

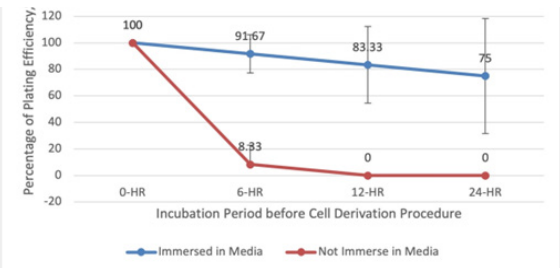

Figure 4: Percentage of plating efficiency based on based on four different incubation time points at room temperature.

Pearson's Chi-square test

This study evaluated the success of cell derivation from postmortem muscle tissues under two conditions: retained in holding media and retained without media. Table 2 presents the Pearson's Chi-square test results evaluating the association between postmortem derivation at different time points and the success rate, based on the percentage of plating efficiency. For the group retained in holding media, the result was not statistically significant, with a p-value greater than 0.05. Therefore, the null hypothesis could not be rejected, indicating no significant association between the time of derivation and the success rate. In contrast, the group that was left without media showed a statistically significant result, with a p-value less than 0.05. This allowed for the rejection of the null hypothesis, indicating an association between the timing of derivation and the success rate.

Table 2: Pearson's Chi-squared test.

	X^2 value	Df (N = 48)	*p*-value
Retained in Media	3.81	3	0.283
Retained without Media	43.36	3	< 0.001

DISCUSSION

Tonicity may play an important role in influencing the successful cell derivation process. Tonicity is the capability of a solution to cause an effect of the cells' volume by regulating the movement of water across the cell membrane (Maldonado & Mohiuddin, 2023). Commonly, there are three conditions related to the tonicity which are isotonic, hypotonic and hypertonic solutions. Isotonic condition is an environment where the osmotic pressure inside and outside of the cells are similar or balanced. In comparison with the hypotonic and hypertonic, an isotonic solution does not cause cell swelling or shrinkage which indicates the zero net movement of water (Debnam, 2008). This condition is hypothesised to minimise the osmotic stress and supports the cell health. While, the hypotonic condition is an environment where the extracellular osmotic pressure is lower compared to the intracellular solute concentration (Lopez & Hall, 2023). The water from the extracellular enters the cell that affect the cells to swell and cause lysis afterwards. It differs to the hypertonic solution where water from intracellular would come out and cause the cells to be shrunk and dies. This occurs since a hypertonic solution contains a higher concentration of solutes compared to the solute concentration inside the cell (Lopez & Hall, 2023).

In the process of deriving the primary cells, it is necessary to ensure an appropriate tonicity. Ignoring the tonicity factors may disrupt the cellular homeostasis by altering the cell membrane integrity and impair the functionality of the enzymes. Intracellular homeostasis is fundamental to tissue and organ stability, providing a supportive environment for efficient communication and real-time coordination among organs (Li et. al., 2024). Consecutively, it will reduce the rate of cell derivation success. It is postulated that optimising tonicity according to cell type and derivation method could improve viability, proliferation, and subsequent cell line establishment. In this context, holding media may act as an isotonic solution, temporarily preventing tissue desiccation without causing chemical alteration. It is because holding media are considered as holding agents that they temporarily prevent tissue desiccation without causing chemical alteration (Singhal et. al., 2017). Conversely, dehydration in tissues retained without media is hypothesised to accelerate cellular damage, potentially through increased reactive oxygen species. It has been reported that dehydration induces cellular damage (potentially through increased reactive oxygen species) in cells, tissues and organs (Xiao & Miwa, 2022).

When an organism dies, the body will stop moving and breathing which contributing to the decomposition from time to time. Decomposition starts about 4 minutes after death with autolysis, during which cells are gradually destroyed, releasing and degrading their components and metabolites (Zapico & Adserias-Garriga, 2022). Decomposition is a continuous process that classified into five stages which are fresh, bloated, decay, post-decay and skeletal (Goff, 2009). This study focused on tissues collected from the animal before reaching the bloated stage of decomposition. Time plays an important role in ensuring the succeeding the cell derivation process from the tissues that have been collected. This study found that the muscle tissues are successfully derived but showing a declining pattern but the data is not significant. This suggests that, for tissues preserved in holding media, timing may not significantly influence derivation success. No sufficient evidence to conclude the differences between the groups are statistically significant. Therefore, it is postulated that other factors in the holding media may contribute to the successful derivation of cells.

In contrast, when the muscle tissues retained without media at room temperature shows that declined pattern with no growth for tissues that have been processed after 12 hours post death. The observed differences between the groups are statistically significant which implies there is an association between the time factor and the success rate. This indicates that time significantly influenced the success of cell derivation when tissues were not protected by holding media. These differences might be due several reasons including the enzymatic and biochemical degradation that starts soon after death (Shedge et. al., 2023). Matrix metalloproteinases (MMPs) are a large family of zinc-dependent enzymes that break down various components of the extracellular matrix (ECM) (Curran & Murray, 1999; Cabral-Pacheco et. al., 2020). They play a key role in ECM remodeling and are grouped into types such as collagenases, gelatinases, stromelysins, and membrane-type MMPs, based on their specific substrates (Curran &

Murray, 1999). It is postulated that holding media may delay autolytic and enzymatic processes, minimising the impact of time on tissue quality and reducing statistical differences between groups. Conversely, in the absence of holding media, autolytic and enzymatic processes may accelerate, leading to greater variability and statistically significant differences. Although this study provides insights into the role of conditioned media in preserving tissue viability, the mechanisms underlying these effects remain speculative. Future research should aim to experimentally validate the influence of tonicity, pH, nutrient composition, and enzymatic activity, including MMP-mediated degradation, on tissue preservation and primary cell derivation. Such investigations could refine tissue retention protocols, improve cell derivation efficiency, and contribute to standardised laboratory practices that are both effective and aligned with sustainable research principles. Nonetheless, plating efficiency provides only limited resolution. Therefore, future studies should incorporate more quantitative and reproducible methods, including histological staining and viability assays, to strengthen the accuracy of morphological and viability assessments.

Roadkill represents a significant example of human-wildlife conflict, impacting biodiversity and contributing to the potential extinction of wildlife species while also posing hazards to human life. As urbanisation and infrastructure continue to expand, effective transportation planning and wildlife management are essential to reducing wildlife–vehicle collisions and conserving biodiversity (Blais et. al., 2024). However, instead of allowing these unfortunate events go unutilised, biological samples can be respectfully collected from the carcasses of the endangered species for valuable research and biobanking purposes. Such studies face challenges, primarily due to uncertainty in determining the time of death of endangered wildlife, complicating sample collection and cell derivation processes. The decomposition process itself is highly variable and influenced by physical factors such as temperature, humidity, and environmental conditions, as well as the activity of other animals (Berg & McClaugherty, 2014; Piaszczyk et. al., 2022).

CONCLUSION

In conclusion, this study demonstrated that Balb/C mice tissues retained in conditioned media at room temperature for up to 24 hours maintained a plating efficiency of approximately 75%. In contrast, tissues without media showed a marked decline in viability, with plating efficiency declining to 8.33% at just 6 hours post-mortem and continuing to decrease thereafter. While tissues in media exhibited no statistically significant difference in viability over time, tissues without media experienced a significant time-dependent decline. These findings highlight the importance of maintaining tissue hydration and stability that potentially influenced by factors such as pH and nutrient availability that play role in supporting cell viability and successful derivation. Further research into these contributing factors could help optimise tissue retention protocols and promote more sustainable and efficient laboratory practices aligned with planetary health goals.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

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