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VIABILITY OF FRESHLY PREPARED CELLS DERIVED FROM FOETAL BRAIN OF RABBIT AT DIFFERENT TEMPERATURE SETTINGS DURING TRANSPORTATION

Redzuan Nul Hakim Abdul Razak[1,2], Muhammad Alif Mazlan[1,2], Azmir Ahmad[1,2], Mohd. Azri Abd. Jalil[1,2], Abdul Halim Abdul Jalil[3] and Muhammad Lokman Md. Isa[1,2]*

[1]*Department of Basic Medical Science for Nursing, Kulliyyah of Nursing, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia.*
[2]*IIUM Molecular and Cellular Biology Research Cluster, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia.*
[3]*Makmal Perintis Sdn. Bhd., Kuala Lumpur, Malaysia.*

*Corresponding Author: lokman@iium.edu.my

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Abstract

The characteristics of rabbit cells, i.e., high proliferative activity, high passage numbers, and morphologically similar to human cells renders it a potential source of a cell line. Nonetheless, they are not widely characterised for research purposes. This study aimed to observe the effects of different temperature settings on the viability of rabbit foetal brain (RFB) cells at different time points. The harvested RFB cells were divided into room temperature (RT), ice packs (ICE), and refrigerator (4 °C) groups. Cell viability was determined at 0, 6, 12, 24, 48, and 72 h. Following cell viability determination, the cells were recultured for 24 h to observe cell viability and morphology post-transportation. The cell viability was analysed statistically for within and more than 24 h duration using the one-way ANOVA test, and the cell morphology was observed under the microscope. For the within 24 h analysis, the ICE group showed the best condition for 24 h transportation due to significantly higher cell viability (p<0.05) compared to the RT group. Meanwhile, for the more than 24 h analysis, the RT group showed the best condition for more than 24 h transportation due to higher cell viability compared to the ICE and 4 °C groups, albeit the non-significance. The reculturing of RFB cells showed no remarkable difference in the cell viability and morphology in all transportation time points at all temperature settings. In conclusion, the study found that the RFB cells are suitable to be transported using ice packs for transportation within 24 h and room temperature for transportation of more than 24 h.

INTRODUCTION

Cells, particularly mammalian cells, are widely used in medical research for various purposes, from basic medical science, like understanding the behaviour of diseases, to applied medical science, like the development of vaccines, drugs, or other therapeutic agents [1,2]. The observation of cellular morphology and cellular capabilities on survival and proliferation is essential before embarking on cellular studies, especially for primary cells, as it might be slightly different from the *in vivo* environment. Thus, the conditions of cell culture need to be optimised to mimic the conditions of the body environment to produce a reliable outcome [3].

Generally, stem cells, primary cells, and cell lines are the standard tools used in cell culture, where each of them has its advantages and physiological function such as anti-inflammatory, dead cells substitution, immunomodulatory action, and maintaining homeostasis depending on the purpose of the studies [4,5]. Among those cells, primary cells represent the best experimental tools that are comparable to

in vivo cellular physiology, albeit they are to culture and exhibit certain limitations on proliferation and differentiation due to their normal cellular physiology [3–6]. Primary cells are procured freshly from human/animal tissues and retain their morphological and physiological characteristics in culture media, similar to their original characteristics *in vivo* [6]. Primary cells retain their genotypic and phenotypic authenticities, allowing the findings from primary cell studies to be precisely translated *in vivo* [1,4,8,9]. Primary cells are not only used in cellular researches but also in cell therapy and immunotherapy [9–11]. Thus, the market supply for primary cells is vital to fulfilling the increasing demand for cell therapies.

The main limitation of cell therapy products is the limited life span of the cells themselves. Additionally, challenges in revitalising and maintaining the primary cells in culture media with a suitable environment also exist. The phase of the cells being stored and transportation are also essential parameters for ensuring a successful embarkment of a study or therapy [12]. As a result, the cells should be manufactured or designed to be stored for some time until it arrived at the healthcare facility and administered to the patient that usually occurs in h to days. This "cold-chain" storage management is often overlooked by the manufacturer, courier, or even the researcher that could hinder the effectiveness and economic output of a promising cell therapy product [13].

During the transportation of the cell product, a few variables should be considered, such as chemical exposure, temperature, time, and type of container since these could affect the quality and clinical effect of the cells [14,15]. These variables have been demonstrated to change cell viability, proliferative rate, potency, morphology, and metabolism [14–16]. Therefore, a well-controlled transport condition should be maintained to preserve the quality of the cells.

Aside from mice and primates, the rabbit is regarded as another potential source of cells for various medical purposes, as it showed high proliferation activity, high passage numbers, and exhibited similar cell colony morphology as human cells [17]. Thus, the study aimed to observe the effects of different temperature settings on the viability of primary cells derived from rabbit foetal brain (RFB) within 24 h and longer transportation duration as a potential new class of cells for laboratory and clinical utility purposes. The study also aimed to observe the recoverability of the RFB cell viability at each transportation time point.

MATERIALS AND METHODS

Ethical Approval

The ethical approval for this study was obtained from the International Islamic University Malaysia (IIUM) Animal Care and Use Committee (I-ACUC) (IIUM/IACUC-2019 (7)).

Study Design

In this study, the RFB cells were divided into three experimental groups based on three different conditions: room temperature with air-conditioning at 24 °C (RT), icebox filled with ice packs (ICE), and refrigerated at 4 °C (4 °C) groups. Six tubes of RFB cells were allocated for each group, where each tube represents a one-time point of transportation h, i.e., 0, 6, 12, 24, 48, and 72 h. The cell viability will be measured at each time point. After the measurement of cell viability, the remaining cells were tested on their recoverability at each time point by reculturing the cells. The cell viability and morphology of the recultured cells were measured and captured, after 24 h incubation. The flow chart of the study design is illustrated in Figure 1.

Foetal Brain Tissue Procurement

The rabbit foetuses were confirmed using an ultrasound scan at four weeks after successful mating. The pregnant rabbit was sacrificed humanely using the exsanguination technique, and the foetuses were obtained by dissecting the womb of the sacrificed pregnant rabbit. The foetuses were placed on ice, and the foetal brain was procured by dissecting the skull of the foetuses. The foetal brain was kept in a pre-mixed commercial culture media (Dulbecco's Modified Eagle Medium [DMEM], Nacalai Tesque, Japan), 10% foetal bovine serum (FBS, TICO Europe, Netherlands) and 1% antibiotic-antimycotic mixed stock solution (Nacalai Tesque, Japan) before cell derivation.

Cell Derivation and Culturing the Cells for Cell Viability Test

The foetal brain was minced into small pieces using a scalpel in 3 mL of Accutase™ (Nacalai Tesque, Japan). The minced tissue was aspirated into a 15 mL conical tube and kept at room temperature for 15 min. Then, the commercial culture media was added into the conical tube and centrifuged for 3 min at 1000 rpm. The supernatant was discarded, and the pellet was re-suspended with fresh commercial culture media. The suspension was transferred into a 12-well plate and incubated in the AutoFlow NU-4750 CO_2 incubator (NuAire, United States) with 5% CO_2 at 37 °C. Once the RFB cells reached 70% confluency, the cells were passaged into a T25 culture flask, followed by passaging into a larger T75 culture flask. Upon reaching 70% confluency, RFB cells in the T75 culture flask were harvested using Accutase™ (Nacalai Tesque, Japan) and divided into respected experimental groups for cell viability testing using an automated cell counter LUNA-II™ (Logos Biosystem, South Korea).

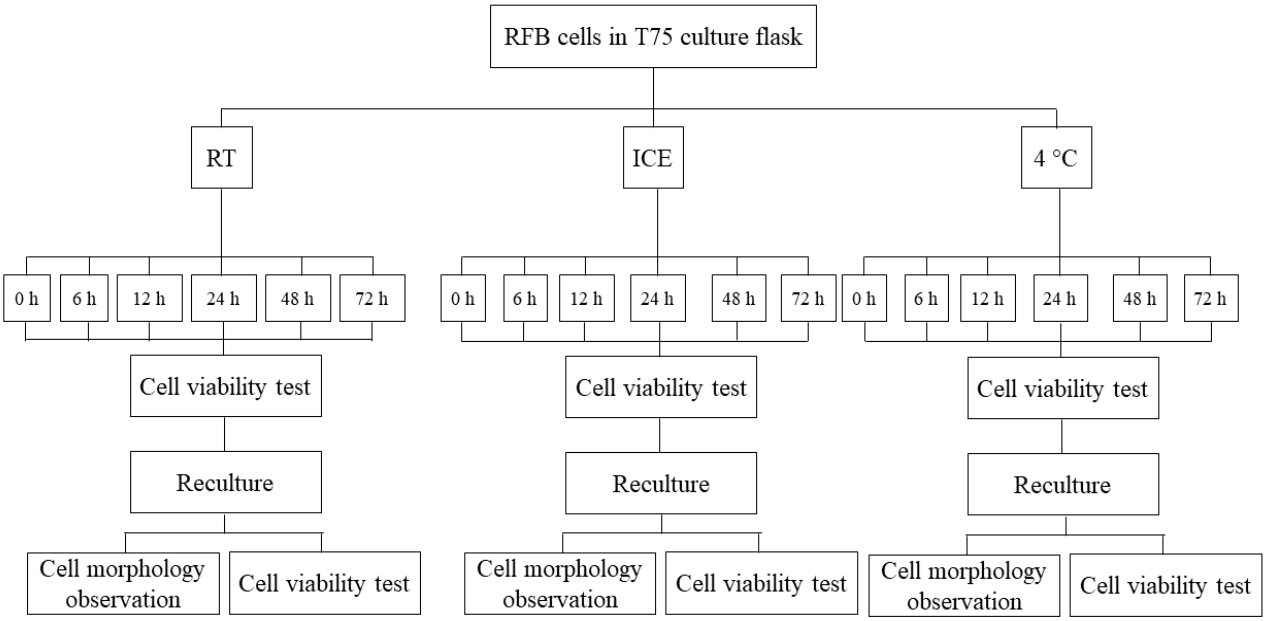

Figure 1. The flow chart of the study design.

Reculturing of RFB Cells at Each Time Point

The RFB cells were recultured at each time point in commercial culture media and incubated in the AutoFlow NU-4750 CO_2 incubator (NuAire, United States) with 5% CO_2 at 37 °C. After 24 h, the cells were observed under an inverted microscope (EVOS^{TM} XL Core Imaging System, Thermo Fisher Scientific, United States) and the images of the cells were captured. Then, the cell viability of the recultured RFB cells was measured using the automated cell counter LUNA-II^{TM} (Logos Biosystem, South Korea).

Cell Viability Test

Before the viability test using the automated cell counter LUNA-II^{TM} (Logos Biosystem, South Korea), 10 µL of the sample was aspirated from each tube of the experimental groups and mixed with 10 µL of 0.4% trypan blue stain (Logos Biosystem, South Korea). Approximately 10 µL of the mixture was dispensed on a consumable slide (Logos Biosystem, South Korea) and inserted into the cell counter. The cell viability was measured in triplicate.

Data Analysis

The results of cell viability were reported as a mean ± standard deviation. The one-way ANOVA test was used to statistically compare cell viability for the intra-group (between time points) and inter-groups (between conditions) analyses. The morphology of cells was captured at each time point of the transportation period and 24 h after reculturing to compare the consistency of cell morphology during transportation and post-reculturing.

RESULTS AND DISCUSSION

Cell Viability in Three Different Conditions Within 24 h

The results of cell viability analysis in three different conditions within 24 h are illustrated in Figure 2. The intra-group analysis for the RT and 4 ℃ groups showed a significant reduction (p<0.05) in the RFB cell viability at 12 and 24 h compared to 0 h, respectively. Meanwhile, no significant reduction is detected in the RFB cells viability up to 24 h of transportation time in the ICE group. The non-significant reduction showed that the RFB cells could maintain their high viability during transportation within 24 h using ice packs. Meanwhile, the inter-group analysis showed that the viability of RFB cells in the ICE and 4 ℃ groups are significantly higher (p<0.05) compared to the RT group within 24 h of the transportation period. Besides, a comparison on the cell viability between the ICE and 4 ℃ groups showed that the viability of RFB cells in the 4 ℃ group is significantly lower (p<0.05) compared to the ICE group at 6 and 12 h, indicating the less appropriate transportation of the RFB cells at 4 ℃ condition. Thus, ice packs are the best medium for transporting RFB cells within 24 h.

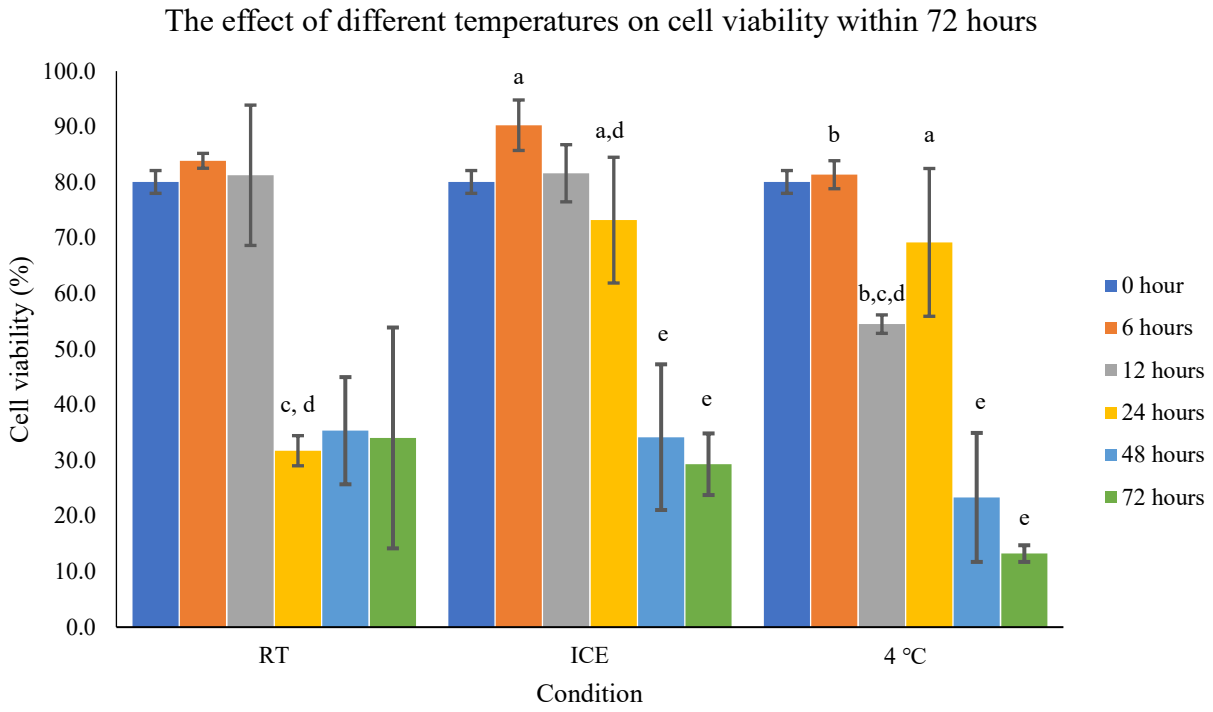

Figure 2. Bar chart showing the percentage of cell viability exposed to three different conditions within 72 hours. Statistical analysis was performed using one-way ANOVA. Data are presented as mean±SD.
(a) Significant difference as compared with the RT group at $p<0.05$; (b) Significant difference as compared with the ICE group at $p<0.05$; (c) Significant difference as compared with the 0 hour group at $p<0.05$; (d) Significant difference as compared with the 6 hours group at $p<0.05$; (e) Significant difference as compared with the 24 hours group at $p<0.05$

Previous studies showed that cells require the specific optimum temperature to be highly viable, and different cells might have different durability at different temperatures [18,19]. Hunt [20] emphasised the necessity to optimise the cryopreservation protocol for different cell types, where some cells can withstand very low temperatures but not others. It implies that different cells have their optimal condition to maintain high viability, and this condition needs to be optimised specifically for different types of cells. In this study, the results demonstrated that the RFB cells showed higher viability when transported on ice packs up to 24 h compared to 4 ℃ and RT. Many studies revealed that most cells showed high viability when they were transported at ambient temperature [21–23]. However, some cells could endure low temperatures for 1 or 2 days, like SaOS2 and Hela cells [22]. The high viability of cells at low temperatures has also been reported by Wheatly and Wheatly [23] and Yu et al. [24], albeit the study used a specialised transport medium for transportation. Studies have proven that different cells require a specific temperature to retain their high viability during transportation. The current study has shown that ice packs were suitable conditions for transporting RFB cells.

Cell Viability in Three Different Conditions for More Than 24 h

The study also attempted to observe the viability of RFB cells for more than 24 h, i.e., 48 and 72 h during long-distance transportation. For this purpose, the viability of RFB cells at 24 h was included in the inter- and intra-group analysis as initial viability to compare the changes of RFB cell viability at 48 and 72 h in three conditions.

The results of cell viability analysis in three different conditions for more than 24 h are illustrated in Figure 2. The intra-group analysis showed that the changes in RFB cell viability in the RT group from 24 to 72 h are not significant, indicating the stable viability of RFB cells at room temperature for more than 24 h transportation. On the other hand, the reduction in RFB cell viability in the ICE and 4 ℃ groups is significant ($p<0.05$) from 24 to 72 h, indicating that the reduced viability of the RFB cells, when transported on ice packs and at 4 ℃ conditions, need to be considered seriously. Meanwhile, the inter-group analysis showed that the RFB cells in the RT group have higher viability at 48 and 72 h compared to the ICE and 4 ℃ groups, albeit the insignificant results. The results also showed that the number of RFB cells in the RT group reduced slightly from 48 to 72 h compared to the larger reduction in the ICE group,

followed by the 4 ℃ group. Thus, transportation of RFB cells at room temperature is the best option for the duration of more than 24 h, as the analysis showed stable RFB cell viability from 24 to 72 h.

The high viability of individual cells at ambient temperature for a prolonged duration has been reported in a previous study by Wang et al. [22]. Meanwhile, the use of specialised transport medium also helped in maintaining cell viability during transportation [21,23]. In this study, a higher percentage of RFB cells were observed at the duration of more than 24 h at room temperature using standard DMEM culture media as compared to ice packs and 4 ℃ conditions.

Recoverability of RFB Cell Viability After 24 h of Reculturing

At each transportation time point, we also recultured the RFB cells for 24 h to observe the recoverability of RFB cell viability at the respected transportation time points. The reculturing of RFB cells at 6, 12, 24, and 72 h of transportation time showed no significant difference in the percentage of viable RFB cells between the RT, ICE, and 4 ℃ groups. The percentage of viable cells is within the range of 9.20 ± 9.91% and 31.73 ± 11.72% (data not shown). The results showed that the RFB cells could be recultured at the same rate after transportation, regardless of the temperature during transportation. Nevertheless, the temperature that can retain high cell viability is the priority in selecting the best temperature for transportation. However, at 48 h of transportation, the cell viability in the RT and ICE groups is significantly higher (p<0.05) than the 4 ℃ group, as shown in Table 1. We speculated that at 48 h, the RFB cells in the 4 ℃ group might undergo a significant reduction in their recoverability compared to the RT and ICE groups. This is supported by a study that reported low temperature might decrease the growth rate of specific cells [25].

Table 1. Cell viability percentage in three different conditions after 24 h of reculturing the RFB cells at 48 h of transportation time.

Cell viability (%)		
RT	ICE	4 ℃
57.10±0.00[c]	50.00±0.00[c]	32.93±12.24

[c] Significant difference as compared with the 4 ℃ group at p<0.05

The captured images of RFB cells under the microscope after 24 h of reculturing showed that the RFB cells have a fibroblast-like shape, as illustrated in Figure 3, Figure 4 and Figure 5. Comparing the gross morphology of RFB cells at 0 h with other transportation time points, no changes were observed in the shape of the cells in all groups. However, the RFB cells in the 4 ℃ group displayed a notable reduction in the number of cells visually from 24 to 72 h. It might indicate a reduction in the recoverability of RFB cell viability after being transported at 4 ℃, although an appropriate quantitative proliferation test is required to produce a more conclusive result.

The results of this study on RFB cells are in agreement with previous studies that reported the decreased growth rate of certain cells at low temperature, while mild heat stress encouraged the proliferation and development rate of cells [25,26]. Moussa et al. [18] and Olson et al. [19] showed that cells required specific optimal temperature, i.e., not too low and not too high temperature to gain high viability [18,19]. In this study, in terms of the recoverability of the RFB cell's viability, room temperature and ice packs were found as the optimal conditions for RFB cells to gain high viability recovery.

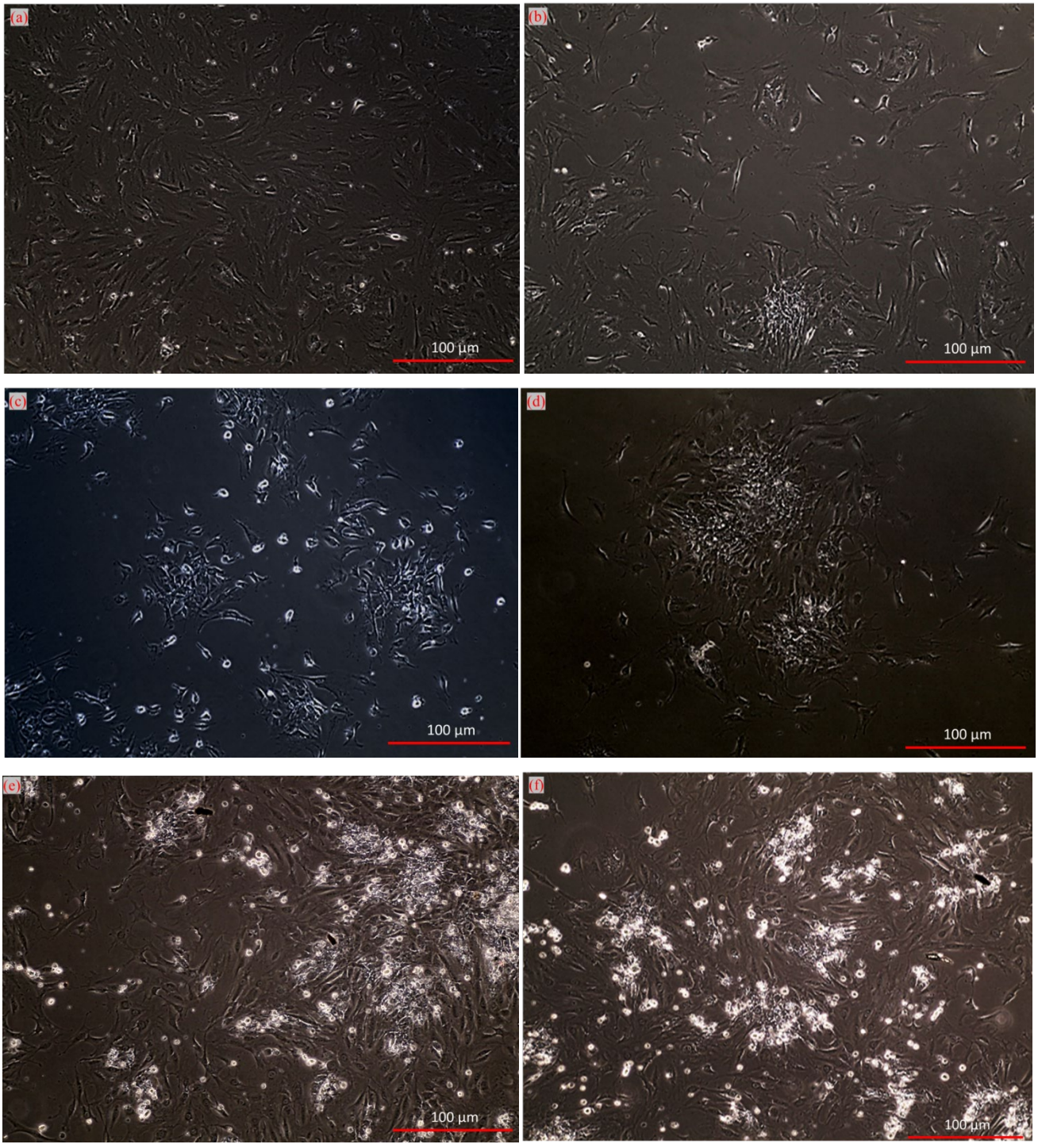

Figure 3. Photomicrographs of RFB cells cultured at room temperature for different time points (×10). **(a)** 0 hour; **(b)** 6 hours; **(c)** 12 hours; **(d)** 24 hours; **(e)** 48 hours; **(f)** 72 hours.

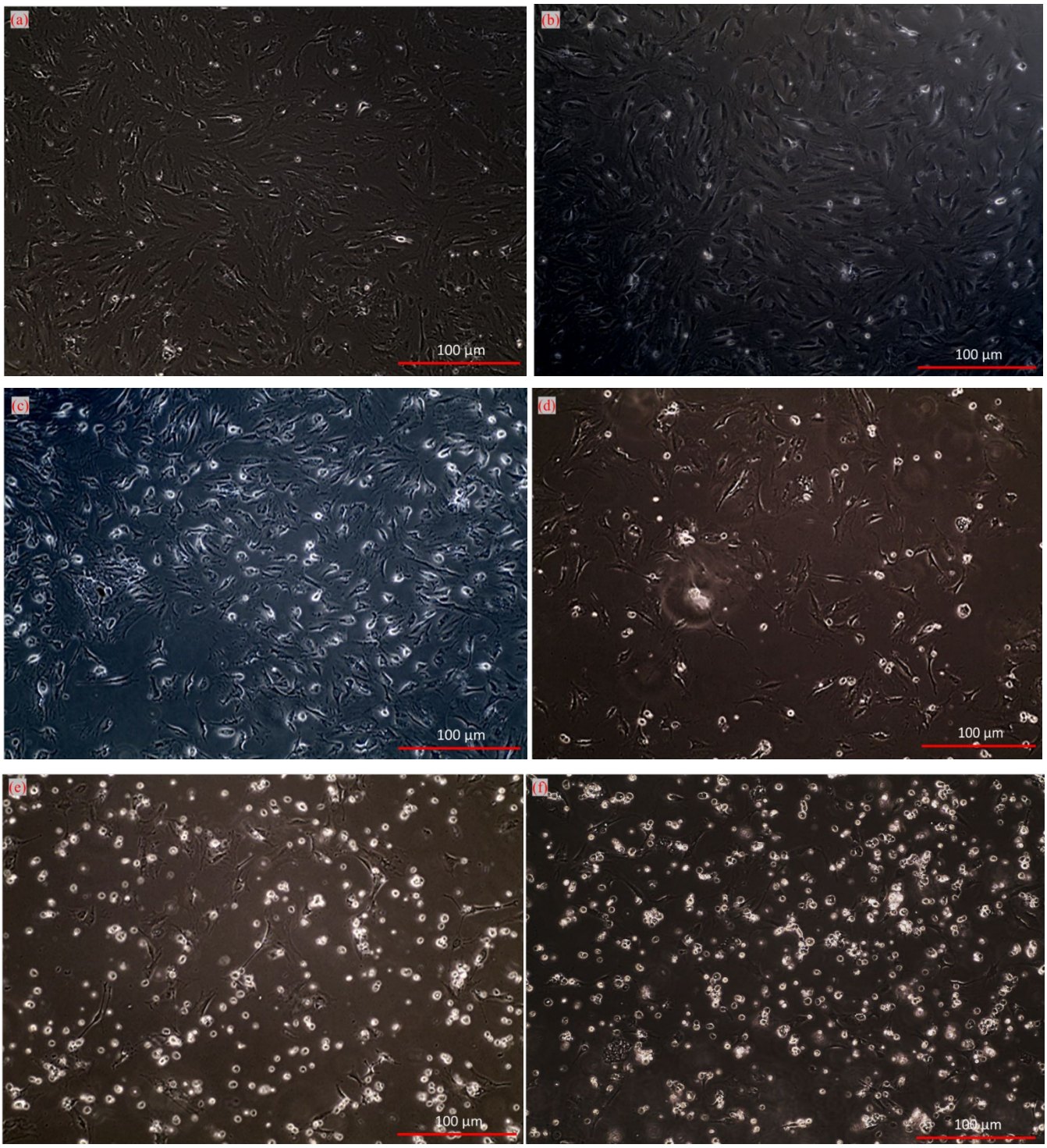

Figure 4. Photomicrographs of RFB cells cultured at 0 ℃ for different time points (×10). **(a)** 0 hour; **(b)** 6 hours; **(c)** 12 hours; **(d)** 24 hours; **(e)** 48 hours; **(f)** 72 hours.

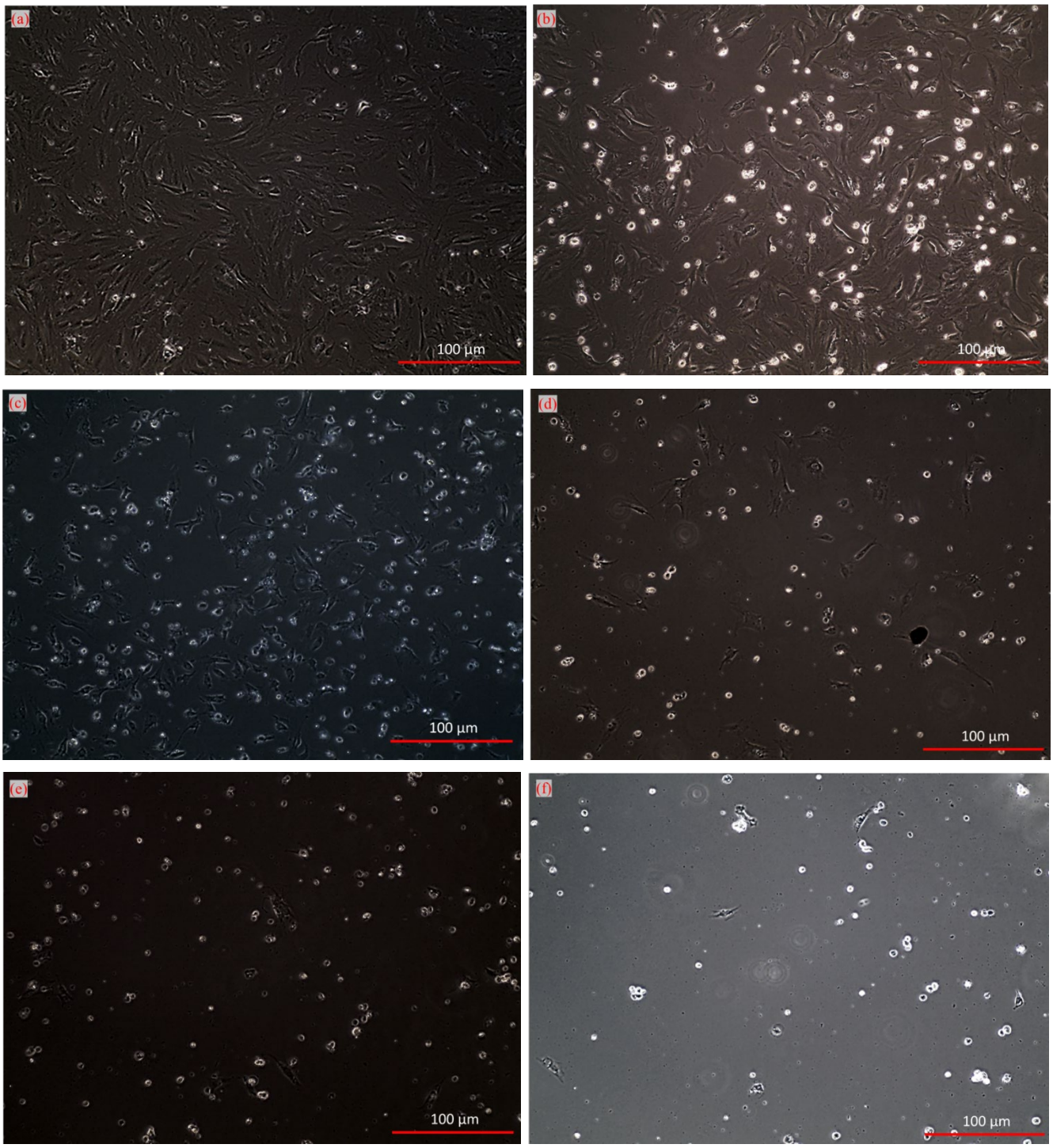

Figure 5. Photomicrographs of RFB cells cultured at 4 ℃ for different time points (×10). **(a)** 0 hour; **(b)** 6 hours; **(c)** 12 hours; **(d)** 24 hours; **(e)** 48 hours; **(f)** 72 hours.

CONCLUSION

This study is the first study that reported the sensitivity of RFB cells towards temperature at specified time exposure for transportation purposes. The study identifies that the transportation of RFB cells using ice packs is a suitable and more economical method for transportation within 24 h in retaining the high viability and recoverability of the cells. However, for a transportation period of more than 24 h, room temperature is better than ice packs in retaining the high viability and recoverability of the RFB cells, which makes it a more economical and straightforward method. Thus, the temperature for transportation of RFB cells depends on the duration of transportation. The current study could also characterise the behaviour of RFB cells concerning the temperature and exposure time duration. The understanding of cell behaviour towards the environment is essential in managing the cells effectively.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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