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Original Article

Cell detachment rates and confluence of fibroblast and osteoblast cell culture using different washing solutions

Taxas de desprendimento celular e confluência de cultura celular de fibroblastos e osteoblastos a partir de diferentes soluções de lavagem

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Abstract

The advancements in the cell culture studies have led to the development of regenerative medicine concept. The aim of this study is to compare the effectiveness of some washing solutions, including phosphate buffered saline (PBS), sodium chloride (NaCl), and ringer's lactate (RL) on the rate of detachment and confluency in fibroblast and osteoblast cell culture. Baby Hamster Kidney 21 clone 13 (BHK21/C13) fibroblast cells and 7F2 osteoblast were cultured on T25 flasks for 3-4 days. Three treatment groups were classified on the basis of different washing solutions used in the moment before trypsinization: PBS, 0.9% NaCl, and RL. Each group was measured for the detachment rate and cell confluence. The measurement was done in 2 passage numbers. The use of PBS, NaCl, and RL washing solution showed that detachment time was less than 5 minutes for the fibroblasts and 3 minutes for the osteoblasts. There was a significant difference in the rate of fibroblast cell detachment ($p=0.006$) and osteoblast ($p=0.016$). The capability of fibroblasts and osteoblasts to achieve a confluence of 10^6 cells/well on the first and second measurements was almost the same between the washing solution groups. The use of physiological 0.9% NaCl solution as a washing solution in fibroblast and osteoblast cell culture has almost the same effectiveness as PBS to help accelerate cell detachment in less than 5 minutes without influencing the capability of cells to proliferate.

Keywords: cell culture, cell proliferation, detaching cell, medicine, washing solution.

Resumo

Os avanços nos estudos de cultura de células levaram ao desenvolvimento do conceito de medicina regenerativa. O objetivo deste estudo é comparar a eficácia de algumas soluções de lavagem, incluindo solução salina tamponada com fosfato (PBS), cloreto de sódio (NaCl) e lactato de ringer (RL) na taxa de desprendimento e confluência em cultura de células de fibroblastos e osteoblastos. Células de fibroblastos Baby Hamster Kidney 21 clone 13 (BHK21/C13) e osteoblastos 7F2 foram cultivadas em frascos T25 por 3-4 dias. Três grupos de tratamento foram classificados com base nas diferentes soluções de lavagem utilizadas no momento anterior à tripsinização: PBS, NaCl 0,9% e RL. Cada grupo foi medido para a taxa de desprendimento e confluência celular. A medição foi feita em 2 números de passagem. O uso de solução de lavagem PBS, NaCl e RL mostrou que o tempo de descolamento foi inferior a 5 minutos para os fibroblastos e 3 minutos para os osteoblastos. Houve uma diferença significativa na taxa de desprendimento de células fibroblásticas ($p=0.006$) e osteoblasto ($p=0.016$). A capacidade de fibroblastos e osteoblastos de atingir uma confluência de 10^6 células/well na primeira e segunda medições foi quase a mesma entre os grupos de solução de lavagem. O uso de solução fisiológica de NaCl (0.9%) como solução de lavagem em cultura de células de fibroblastos e osteoblastos tem quase a mesma eficácia que o PBS para ajudar a acelerar o desprendimento celular em menos de 5 minutos sem influenciar a capacidade das células de proliferar.

Palavras-chave: cultura celular, proliferação celular, célula destacada, medicamento, solução de lavagem.

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1. Introduction

A cell-based (in vitro) assay is a crucial step in the new drug invention process (Langhans, 2018). In the development and invention of new drugs, a lot of researchers apply cell culture to determine protein expression, genetic changes and metabolic process of cells (Chengalvala et al., 2007; Kim et al., 2020). Cell culture is generally used for assay as a preliminary drug screening that offers a rapid result and relatively inexpensive. The utilization of the assay in culture systems has various advantages including the ability to control the environment (pH, temperature, osmotic pressure, O_2, and CO_2), and physiological conditions (Verma et al., 2020).

Research in the discipline of biomolecular dentistry is rapidly growing, particularly in cell culture systems. Cell culture are often applied using primary cells or adherent cell lines (Segeritz and Vallier, 2017; Nascimento et al., 2021), for examples fibroblasts and osteoblasts. Fibroblasts are one of most essential parts in the pulp, periodontal ligament and gingiva (Otabe et al., 2012). The cells that were cultured in a flask/petri dish should attain a confluent number before the assay. Furthermore, cell detachment is a process of cells passage into a new flask by trypsinization (Mótyán et al., 2013).

Trypsinization is a popular technique of detaching cells although it has the disadvantage of decreasing cell viability due to causing damage to membranes and extracellular matrix. It takes longer to reach confluence in cells with low viability (Tsuji et al., 2017; Kurashina et al., 2019). Some researchers reported that trypsin has a toxic effect if it is rested in the media for a long time and if it is exposed to fluorescent light (Canavan et al., 2005; Huang et al., 2010; Danhier et al., 2013). Recently, there are several methods to accelerate the trypsinization process including the provision of direct electric current. The utilization of DC electric current would cause changes in Ca^{2+} ions in the cell. Stimuli particularly electrical stimuli could influence cell properties and functions including adhesion, arrangement of the cytoskeleton, proliferation, growth factor and gene expression, and cell viability (Sun et al., 2006; Chen et al., 2019; Leppik et al., 2020). Another common method of trypsinization is the use of a washing solution such as phosphate buffer saline (PBS) immediately before trypsin is given in order to increase the trypsin effectiveness and to ease the cells separation from the base of the culture flask (Patil et al., 2020).

PBS is the most commonly used biological buffer which is an isotonic solution capable of maintaining osmolarity with no toxicity to cells. Therefore, it is suitable for washing processes in cell culture (Martin et al., 2006; Chen et al., 2017). PBS is a water-based salt solution containing disodium hydrogen phosphate, sodium chloride, and in some formulations, there is addition of potassium chloride and potassium dihydrogen phosphate. However, other studies have shown that clinical use of PBS as an eye rinse has shown evidence of corneal calcification due to phosphate content (Thorat and Suryanarayanan, 2019).

Sodium chloride (NaCl) 0.9% is another standard physiological solution utilized for numerous purposes of handling animal cells or tissues with the aim of maintaining cell metabolic activity (Li et al., 2016). The use of 0.9% NaCl and ringer lactate (RL) have been introduced for graft preservation. In the US, the utilization of cell culture-based storage solution media has been developed, and this allowed maintaining cell viability for graft (Arabiun et al., 2020). There was no significant difference in chondrocyte survival between MEM-based culture media and Ringer's lactate (RL) based storage solution (Harb et al., 2017). Pharmacologically RL is the same as other isotonic fluids to replace fluid in the extracellular compartment.

The importance of the trypsinization process in cell culture systems would influence the long-term viability of cells and the rate of cell detachment. Therefore, this study aimed to compare the use of physiological solutions 0.9% NaCl and RL against PBS as a washing solution in fibroblast and osteoblast cell culture which is effective and safe.

2. Materials and Methods

2.1. Pre-culture in T75 flask

Two types of cell lines were used in this study, fibroblast BHK-21 C13 (Sigma, 85011433) and osteoblast 7F2 (ATCC, CRL-12557). The fibroblast cell line BHK-21 C13 was cultured on T75 flasks in GMEM (Sigma, G5154), 2 mM Glutamine (Gibco, 35050061), 5% Tryptose Phosphate Broth (Sigma, T8159), 10% FBS (Sigma, 12106C) and 1% penicillin/streptomycin (Gibco, 15140122). While, the osteoblast cell line 7F2 was cultured in αMEM without ribonucleosides, deoxyribonucleosides and sodium bicarbonate (Sigma, M0894), with 10% FBS and 1% penicillin/streptomycin. Cells were incubated at 37°C, 5% CO_2. PBS (Gibco, 20012027) was used regularly as a washing buffer. Cells were routinely passaged in 0.25% Trypsin-EDTA (Sigma, T4049) until reaching 85-90% confluence. A passage number between 47-53 was used in this study.

2.2. Culturing cells in T25 flask

BHK-21 C13 and 7F2 cells were cultured in T25 flasks at a seeding density of 1.25 x 10^5 cells per well. Each sample was done in triplicate and 2 ml of culture medium was used in every well. The cells were incubated at 37°C (5% CO_2) for 3-4 days to reach 85-90% confluence.

2.3. Cell detachment rate analysis

The confluent cells were washed 3 times using 3 different washing buffers: PBS (Gibco, 20012027), 0.9% NaCl (Sigma, S5886) and RL (Sigma, L4263). A volume of 2 ml was used in each well. Afterwards, the washing buffer was removed and replaced with 0.3 ml of 0.25% Trypsin-EDTA (Sigma, T4049). Cells were placed in an incubator (37°C, 5% CO_2) for the detachment process. The cell detachment was observed using light microscopy in 3-5 minutes until fully detached.

2.4. Confluence cell analysis

When the cells were fully detached, 2 ml of the culture medium was added into the cells to inhibit further tryptic activity. The cell suspensions were then centrifuged at 100xg

for 5 minutes. The supernatant was removed and the pellet was resuspended with culture medium. The number of cells was counted by using 1x Trypan blue (Sigma, T6146) and Haemocytometer (Sigma, BR717810) counting chamber.

2.5. Subculturing (Phase 2)

After performing the washing treatment with 3 different buffers, each group of cells was sub-cultured in a T25 flask. The cell culture and detachment methodology in the second phase was done by following the same procedure as in the previous phase.

3. Results

3.1. The composition of the washing solution on cell culture of BHK21 C13 fibroblasts and 7F2 osteoblasts

In this research, the cell detachment process was carried out using the trypsinization method. The effectiveness of trypsinization for cell detachment could be increased by rinsing the cells with saline solution (PBS) as a control and compared with normal saline (0.9% NaCl) and Ringer's lactate (RL) solutions with their contents (as shown in Table 1).

Table 1. Composition of washing solutions PBS, 0.9% NaCl and RL.

Composition	PBS 7.2 (1x)	0.9% NaCl	Lactate Ringer
Na^+ (mmol/L)	157	154	130
Cl^- (mmol/L)	140	154	109
$[Na^+]$: $[Cl^-]$ ratio	1:1	1:1	1.19:1
K^+ (mmol/L)	4.45	0	4
HCO_3^-/ bicarbonate	0	0	28 (lactate)
Ca_2^+ (mmol/L)	0	0	1.4
Mg_2^+ (mmol/L)	0	0	0
Glucose (mmol/L)	0	0	0
Na_2HPO_4 (mmol/L)	10	0	0
KH_2PO_4 (mmol/L)	1.76	0	0
pH	7.1-7.3	4.5-7.0	4.0-8.0
Osmolarity (mOsm/L)	280	308	295

"0": not available.

3.2. The effectiveness of the washing solution on BHK-21 C13 and 7F2 cell culture

The use of PBS, 0.9% NaCl and RL as washing solutions in the trypsinization process of fibroblast cell culture showed different detachment times. The outcomes of cell observation using an inverted microscope at minute 4 showed a change in the morphology of fibroblast cells from an elongated spindle to a rounded spindle which indicated that the cells were detached from the bottom of the plate. Fibroblast cell culture using PBS and NaCl as washing solutions showed that the entire cells were detached (rounded cells) while in RL, there were abounding cells still attached. Fibroblast cells that are elongated spindle-shaped were more than the rounded fibroblast cells (see Figure 1A, B, C). Fibroblast cell culture that was detached through the trypsinization process was sub-cultured on a new culture flask. The growth of fibroblast cells was observed until the cells reached confluence on day 3 using an inverted microscope. The PBS and NaCl group reached confluence faster than the RL group (see Figure 1D, E, F).

3.3. Detachment rate and confluence in Fibroblast BHK-21 C13 cell culture

There was a significant difference (p=0.006) in the time necessary to release the attachment of fibroblast cells from the bottom plate based on the evaluation of the time required to release them from the bottom plate. The use of PBS as a washing solution prior trypsinization resulted in faster cell detachment than NaCl and RL (as shown in Table 2). The rate of cell detachment in the PBS group did not significantly change from the NaCl group (p=0.821), but it significantly differed in the RL group (p=0.008). The application of RL washing solution resulted in considerably shorter cell detachment time than the NaCl group (p=0.012). In fibroblast cell culture,

Table 2. The effect of different washing solutions PBS, NaCl 0.9% and RL on the average release time and confluence of BHK-21 C13 fibroblast cells.

	N sample	PBS	NaCl	RL	p-value
Detachment rate (minutes)	Triple	4.17 ± 0.29	4.33 ± 0.29	5.83 ± 0.29	0.006*
Confluence (cells/well)					
Phase 1					
Seeding (10^5)	Triple	1.25 ± 0.00	1.25 ± 0.00	1.25 ± 0.00	
Harvest (10^6)		4.23 ± 0.13	3.37 ± 0.09	3.17 ± 0.15	
Phase 2					0.835
Seeding (10^5)	Triple	1.25 ± 0.00	1.25 ± 0.00	1.25 ± 0.00	
Harvest (10^6)		5.57 ± 0.15	4.68 ± 0.16	4.43 ± 0.15	

*: Significantly different on p<0.05; PBS: Phosphate Buffered Saline; NaCl: Sodium Chloride 0.9%; RL: Ringer's Lactate. Phase 1 = 1st confluence test; Phase 2 = 2nd confluence test.

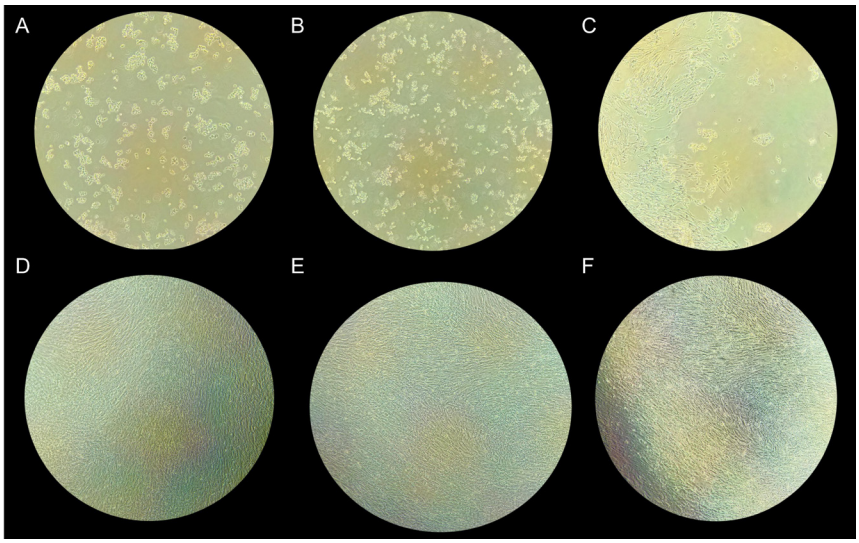

Figure 1. Examination of fibroblast cells with an inverted microscope with 20x magnification. The rate of cell attachments release was observed at minute 4 in the PBS (A), NaCl (B) and RL (C) groups. Fibroblast cell confluence was seen on day 3 in the PBS (D), NaCl (E) and RL (F) group.

the cell detachment rate was sequentially ordered in the PBS<NaCl<RL group (see Figure 2).

Phosphate Buffer Saline, NaCl, and RL washing solutions had no effect on fibroblast cell confluence in phases 1 and 2 (p=0.835) (as shown in Table 2). The ability of fibroblast cells to proliferate was not affected by the cell detachment time up to 5.83 minutes in the RL group, despite its non-significant decrease when compared to the PBS (p=0.833) and NaCl (p=0.901) groups. The ability of fibroblast cells to proliferate and reach confluence on day 3 was similar in the PBS, NaCl and RL group (see Figure 3).

3.4. Detachment rate and confluence in osteoblast 7F2 cell culture

Regardless of the base plate, the results of 7F2 osteoblast cell detachment revealed differences in cell velocity. The morphology of the 7F2 osteoblast cells changed from cuboidal to rounded, as observed under the microscope. The morphology of osteoblast cells has changed, indicating that the cells have separated from the base. In the trypsinization process of osteoblast cell culture, the use of PBS, 0.9%NaCl, and RL as a washing solution resulted in cell detachment in less than 3 minutes. On day 3, the 7F2 osteoblast seeding yield in each group was 80-90% confluent (see Figure 4).

The rate of osteoblast detachment from the base plate was significantly different in the PBS, NaCl, and RL groups (p=0.016). When compared to fibroblast cells, the use of PBS, NaCl, and RL washing solutions on osteoblast cells resulted in a time reduction of 2-2.38 minutes (as shown in Table 3). The NaCl (p=0.019) and RL (p=0.035) groups demonstrated a significant difference in detaching cells from the PBS group. Meanwhile, in osteoblast cell culture, the use of NaCl as a washing solution did not significantly differ from RL (p=0.872) (see Figure 2). The PBS, RL, and NaCl groups had the highest rate of cell detachment in osteoblast cell culture.

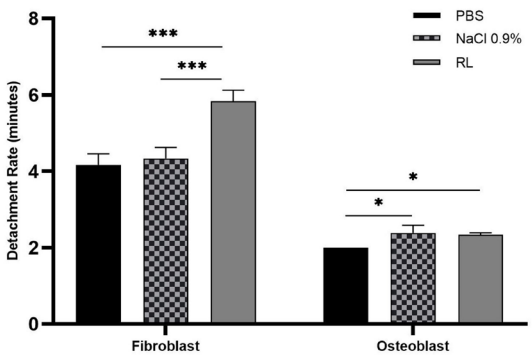

Figure 2. The average time required for fibroblast and osteoblast cells to detach from the bottom plate using PBS, NaCl and RL washing solutions. PBS: Phosphate Buffered Saline; NaCl: Sodium Chloride 0.9%; RL: Ringer's Lactate. ***: significantly different on p<0.001; *: significantly different on p<0.05.

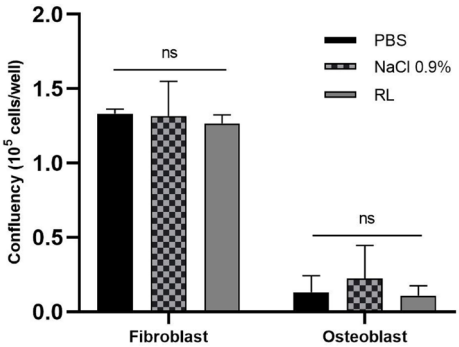

Figure 3. Confluence of fibroblast and osteoblast cell culture in the use of rinsing solutions PBS, NaCl and RL. PBS: Phosphate Buffered Saline; NaCl: Sodium Chloride 0.9%; RL: Ringer's Lactate. ns: not significant on p>0.05.

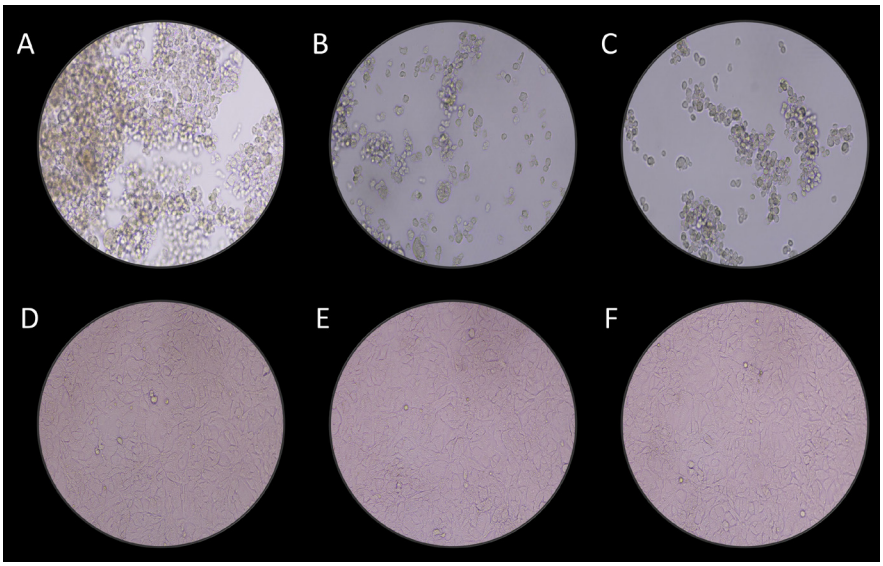

Figure 4. Examination of osteoblasts with an inverted microscope with 20x magnification. The cell attachment release was observed at minute 4 in the PBS (A), NaCl (B) and RL (C) groups. Osteoblast cell confluence was seen on day 3 in PBS (D), NaCl (E) and RL (F) groups.

Table 3. Effect of different washing solutions PBS, 0.9% NaCl and RL on the average detachment rate and confluence of 7F2 osteoblasts.

	N sample	PBS	NaCl	RL	p-value
Detachment rate (minutes)	Triple	2.00 ± 0.00	2.38 ± 0.20	2.33 ± 0.06	0.016*
Confluence (cells/well)					
Phase 1					
Seeding (10^5)	Triple	1.25 ± 0.00	1.25 ± 0.00	1.25 ± 0.00	
Harvest (10^6)		5.93 ± 0.11	5.60 ± 0.13	5.05 ± 0.05	
Phase 2					0.622
Seeding (10^5)	Triple	1.25 ± 0.00	1.25 ± 0.00	1.25 ± 0.00	
Harvest (10^6)		6.06 ± 0.04	5.83 ± 0.09	5.15 ± 0.09	

*: Significantly different on p<0.05; PBS: Phosphate Buffered Saline; NaCl: Sodium Chloride 0.9%; RL: Ringer's Lactate. Phase 1 = 1st confluence test; Phase 2 = 2nd confluence test.

The use of PBS, NaCl, and RL washing solutions had no significant effect (p=0.622) on the level of osteoblast confluence in phases 1 and 2. On day 3, osteoblasts washed with NaCl had a higher confluent than those washed with PBS or RL, while there was no statistically significant difference between NaCl-PBS (p=0.736) and NaCl-RL (p=0.627) (see Figure 3).

4. Discussion

In drug development, cancer research, and stem cell research, cell culture is commonly used as a preliminary drug screening. The majority of cells are now cultivated using two-dimensional (2D) methods, however new and enhanced approaches using three-dimensional (3D) cell culturing techniques provide persuasive evidence that considerably more advanced studies can be performed generating useful insights (Langhans, 2018; Jensen and Teng, 2020). As a result, it is critical to maintain healthy and favourable cell conditions in order to obtain confluent cell culture in a reasonable amount of time (Dumont et al., 2016).

The detachment of adhering MSCs is typically aided by the enzymatic breakdown of adhesion proteins. At various concentrations, trypsin is the most widely utilized detachment agent (0.25%, 0.05%, and 0.025%) (Brown et al., 2007). Most surface proteins are degraded during this operation, and excessive trypsinization can cause lasting cell damage. On the other hand, insufficient trypsinization results in an incomplete cell harvest. The concentration of enzyme, the length, and the temperature (typically 37°C) of the detachment can be tweaked (Freshney, 2011). Several alternatives to animal-free and/or softer detachments have been developed. The use of a washing solution before the trypsinization process is one of the most common ways of improving trypsinization efficiency (Bi et al., 2013). By absorbing calcium ions required for cell attachment

through calcium-dependent adhesion molecules, trypsin containing ethylenediamine tetra-acetic acid (EDTA) can facilitate cell release (Hong et al., 2009).

Sodium chloride, sodium phosphate, potassium chloride, and potassium phosphate are all included in a washing solution like PBS. PBS is an isotonic solution that is non-toxic to cells, and it maintains osmolarity making it ideal for washing processes in cell culture and immunoassays such as ELISA and immunohistochemistry (Martin et al., 2006; Chen et al., 2017). In molecular biology and protein research, PBS is frequently employed as a dilution buffer. It can be taken up to five minutes for cells detachment from the plate when trypsin is used for the detachment. The goal of utilizing PBS is to wash away the media's effect so that trypsin can properly separate cells. Another major goal is to shorten the contact period between trypsin and cells in order to prevent cell toxicity (death) during the trypsinization process (Segeritz and Vallier, 2017).

According to the rate of cell detachment in fibroblast cell culture, PBS was faster than NaCl, whereas NaCl was faster than RL (PBS>NaCl>RL). PBS solution takes 4.17 minutes to perform trypsinization, NaCl takes 4.33 minutes, and RL takes 5.83 minutes. No significant temporal difference in cell detachment was seen when cells were washed with NaCl solution compared to PBS. This means that the NaCl solution is equally effective as PBS as a cell culture washing solution. The NaCl solution's content was identical to that of PBS, with the addition of phosphate buffer and KCl to PBS. This resulted in the same effectiveness. Calcium can be removed from cells involved in intercellular adhesion as well as from the plate's bottom by using PBS or NaCl solutions. These two solutions have no calcium (Ca^{2+}) and magnesium (Mg^{2+}) ions, making it easy to separate the cells (Sorour et al., 2016).

In fibroblast cell culture, the use of RL solution as a washing solution resulted in a longer duration for cell detachment than PBS or NaCl. The cells took up to 5.83 minutes to be separated from their attachments. Ca^{2+} ions are present in the RL solution, whereas Mg^{2+} ions are not. Because of the high concentration of Ca^{2+} ions in RL, washing may make it difficult to separate the cells. In cell-cell and cell-matrix interactions, calcium and magnesium play a crucial role. Before dissociation, PBS devoid of Ca^{2+} and Mg^{2+} is used in cell culture to do the dissociation process. Calcium and magnesium make it possible for cells to adhere together. The longer the trypsinization process takes, the more cell membrane damage occurs reducing availability (Kurashina et al., 2019). The shape of fibroblast cells changed from elongated to rounded spindles, indicating cell detachment. Almost all cells in the PBS and NaCl groups were rounded up to 4 minutes since trypsin administration, however many spindle-shaped cells remained in the RL group. Cell detachment took about 3-5 minutes after trypsinization.

Furthermore, the use of a washing solution on osteoblast cells revealed that cell detachment was faster in the PBS group than in the NaCl group, while NaCl was slower than RL or PBS<RL<NaCl. To put it another way, PBS washing solution is more typically utilized in various cell culture, such as fibroblast and osteoblast culture. It was also discovered that the three solutions (PBS, NaCl, and RL) were capable of removing the effect of FBS protein from cells leading to a quick trypsinization process. The use of RL washing solution on osteoblast cells showed faster cell detachment than its use on fibroblast cells. This is in accordance with research which stated that RL solution can increase the expansion of osteoblast cells in bone marrow stromal cells (BMSCs) (Dias et al., 2019).

It appears that fibroblasts and osteoblasts in each group can multiply exponentially based on cell confluence measurements. In fibroblast and osteoblast cell culture, the use of PBS solution as a washing medium caused no cell membrane damage, which was characterized by mortality and impaired proliferative potential. Fibroblasts can renew and multiply indefinitely. The ability of fibroblasts to proliferate was reduced in the RL-washed group compared to osteoblast cells, albeit the difference in cell counts was not significant. Meanwhile, the NaCl group's ability to grow fibroblast and osteoblast cells was unaffected.

5. Conclusion

In conclusion, we have reported that the use of physiological NaCl solution as a washing solution in cell culture aids cell detachment rate without interfering with the fibroblast and osteoblast proliferative ability. As a result, if researchers are unable to access PBS, NaCl solution can be used as a substitute washing solution as it is relatively inexpensive and easy to be obtained.

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