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RESEARCH ARTICLE

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Utilization of Expired Platelet Concentrate for Production of Human Platelet Lysate as a Medium for T47D Cell Propagation

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Background: Platelet concentrate (PC) has a short shelf life (5 days). Expired PC cannot be used for clinical purposes. PC is used for human platelet lysate (HPL) production, which was found to be more effective than FBS at increasing T47D cell proliferation. HPL production using expired PC has not been reported. This study aimed to investigate whether the use of HPL produced from expired PC (storage duration >5 days) can increase the proliferation of T47D cells *in vitro*.
Materials and methods: Expired PC samples with a shelf life of 7 and 11 days were used to produce HPL via freeze/thaw method. pH, total protein content, glucose and albumin levels were measured. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to measure proliferation rate and doubling time of HPL-treated T47D cells.
Results: After HPL production, the glucose level was influenced by the pH (p=0.003), and albumin level was influenced by total protein content (p=0.030). HPL stored for 7 and 11 days increased cell proliferation rate by 1.41 and 1.80 times higher than 10% FBS, respectively. HPL produced from expired PC did not cause morphological abnormality of the cells. In this study, the glucose levels affected cell proliferation (p=0.030). High glucose levels inhibited T47D cell proliferation.
Conclusion: Expired PC can be used as a potential material for HPL production, since HPL produced from expired PC increases cell proliferation rate and shortens cell doubling time.

Keywords: *cell proliferation, human platelet lysate, platelet concentrate, thrombocyte, T47D*

Introduction

Human platelet lysate (HPL) is one of the media that can be used as an alternative to fetal bovine serum (FBS) or fetal calf serum (FCS), the gold standard medium for cell culture. HPL contains growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor beta 1 (TGF-β1), insulin-like growth factor-1 (IGF-1) and basic fibroblast growth factor (bFGF), as well as proteins that contribute in hemostasis.[1-4] HPL does not contain aggregated platelets, hence does not cause cell clumping. In addition, HPL is also

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xeno-free which reduces immunological reactions for cell therapy.[5] HPL has been used for mesenchymal stromal cells (MSCs)[6], HaCaT[7], Vero, Hep-2[8], and T47D cell culture. HPL was found to be more effective than FBS at increasing T47D cell proliferation, hence shortening the doubling time of T47D cells.[9]

HPL is derived from good quality platelet concentrate (PC), which is freshly collected or stored for 3-5 days. PC is obtained from male donors aged ≤35 years with O blood group. HPL is prepared from PC via repeated freeze/thaw cycles.[9] This process helps to release growth factors from platelets.[10]

According to the Regulation of the Health Minister of Republic of Indonesia No. 91/2015 about the standards of blood transfusion services No. 91/2015, platelets that can be used for treatment have a shelf life of five days.[11] A decrease in pH will occur as the storage time of PC increases, resulting in a decrease in platelet viability. Since PCs have relatively short shelf life, expired PCs are routinely discarded. Therefore, this study aimed to investigate whether the use of HPL produced from expired PC can increase the proliferation of T47D cells *in vitro*. Expired PCs are potential primary material for HPL production compared to freshly isolated PCs, which are more expensive and difficult to obtain.[12]

Materials and methods

Donor Selection Process

PCs used in this study were freshly collected from donors who meet the criteria according to the Regulation of the Health Minister of Republic of Indonesia No. 91/2015. Blood was taken from male donors aged ≤35 years with O blood group. All prospective donors had signed the informed consent forms before having their whole blood drawn using Triple Bags blood bags containing the anticoagulant CPDA-1 (JMS PTE Ltd, Singapore, Singapore). Screening of whole blood for transfusion transmitted diseases (TTD), i.e. Hepatitis C, Hepatitis B, Syphilis and HIV was performed using ARCHITECT chemiluminescent immunoassay (Abbott, Abbott Park, IL, USA). Non-reactive blood was used to produce PCs. PCs that have exceeded their shelf life were obtained from the Indonesian Red Cross (PMI) Yogyakarta. Ethical approval was obtained from the Ethics Commission of the Faculty of Medicine Gadjah Mada University (No. KE/FK/0321/EC/2021).

PC Preparation

Whole blood was centrifuged at 3800 g for 15 minutes at 4°C in a refrigerated blood bag centrifuge (Kubota, Osaka, Japan) to obtain packed red cells (PRC) and platelet rich plasma (PRP). These blood components were then separated using Novomatic plasma extractor (Lmb Technologie GmbH, Oberding, Germany). The PRP was centrifuged at 3000 g for 20 minutes at 22ºC to obtain PC. The PC was collected in a 50-70 mL bag and stored in a plasma agitator (Helmer, Noblesville, IN, USA) at 20-24ºC for 11 days.[11] The PCs used in this study were stored for more than five days. During the storage period, physical and chemical changes, including colour, turbidity, swirling, pH and glucose levels were analyzed.

HPL Production

HPL was produced using the freeze/thaw method. The PC freezing process began with plasma freezing and was followed by thawing in a 37°C water bath. Deep freezing at -80°C was used for the second and third freezing. After that, cell debris was separated from the supernatant by centrifugation at 4000 rpm for 15 minutes. The supernatant was collected under aseptic conditions. pH, glucose and albumin level, and total protein content of each HPL sample were then measured.

Glucose Level Measurement

Ten µL sample was mixed with 1 mL God FS Glucose reagent (DiaSys, Holzheim, Germany). The sample was then incubated for 20 minutes at room temperature. The absorbance of the PC was measured at 546 nm.

Total Protein Measurement

Twenty µL sample was mixed with 1 mL of mono reagent total FS protein (DiaSys, Holzheim, Germany) and incubated for 5 minutes at room temperature. The absorbance of the PC was measured at 540 nm. Standard values were used for measurement of total protein.

Albumin Level Measurement

Ten µL sample was mixed with 1000 µL of Albumin FS (DiaSys, Holzheim, Germany) and incubated for 10 minutes at room temperature. After that, the absorbance of the PC was measured at 540 nm wavelength. The albumin concentration was measured by comparing the standard values.

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Cell Proliferation Assay

T47D cells were obtained from Laboratory of Parasitology, Faculty of Medicine Gadjah Mada University, Indonesia. After reaching the desired confluency, T47D cells were harvested and the density of the cell was measured. The cells were loaded into each well (10,000/100 µL) and incubated overnight in a CO_2 incubator at 37°C. After the incubation period, culture medium was discarded and the cells were washed with PBS. Medium of T47D cells was replaced with Roswell Park Memorial Institute (RPMI) medium containing 2% penstrep, 0.5% fungizone, and 10% HPL. The cells were then incubated overnight in a CO_2 incubator at 37°C. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was used to measure cell proliferation rate.

Cell Doubling Time Analysis

T47D cells were incubated in culture medium containing 5% HPL for 24 hours, 48 hours and 72 hours. The absorption value was measured for each treatment. In addition, the doubling time value for each treatment was calculated by creating a regression graph of cell viability versus incubation time.

Data Analysis

Kolmogorov-Smirnov test was used to check data normality. Furthermore, Pearson correlation test was also performed to determine the relationship between total protein content and albumin level, total protein content with glucose level, and glucose level with cell proliferation. The difference in glucose levels before and after HPL production was analyzed using Wilcoxon Least Significant Difference (LSD) to determine the differences in cell proliferation and doubling time between samples. SPSS 21 (IBM Corporation, Armonk, NY, USA) was used for statistical analyses.

Results

Characteristics of Expired PC

In this study, PC samples were classified into two groups, i.e. PCs with a shelf life of 7 (7D) and 11 days (11D). Each group consisted of three bags of PCs. Before HPL production, the quality of the PC was assessed using several parameters, i.e. the presence or absence of swirling, colour, turbidity level, platelet count, and glucose level. The PCs used in this study were collected from donors aged 20-34 years. In general, PCs stored for more than five days still had good quality. The pH of PCs with a shelf life of 7 days were between 7.15-7.30, while the pH of PCs stored for 11 days were between 6.13-7.20. The average glucose level of PCs stored for 11 days was lower than those that were stored for 7 days (Table 1).

Total Protein Content and Albumin Level in HPL

Sample 11D-3 had the highest total protein in HPL, while sample 7D-3 had the lowest total protein. Glucose levels affected total protein content in expired PCs. Meanwhile, platelet count, pH and age of donor did not affect the total protein content in HPL. Albumin levels in HPL correlated with total protein content and platelet count (Figure 1).

Glucose Level in HPL

Glucose levels in PC and pH influenced glucose levels in HPL. Glucose levels affected the proliferation of T47D cells (Figure 2).

Table 1. Characteristics of expired PCs used for HPL production.

Storage Duration (Days)	Sample	Age of Donor (Years)	PC Parameters					
			Colour	Swirling	Turbidity	pH	Glucose (mg/dL)	Platelet Count ($x10^3$ µL)
7	7D-1	25	Reddish-yellow	Present	Slightly turbid	7.15	363	586
	7D-2	28	Yellow	Present	Clear	7.30	398	584
	7D-3	25	Yellow	Present	Clear	7.30	399	704
Average						**7.24**	**386.7**	**624.7**
11	11D-1	20	Yellow	Present	Clear	7.20	361	315
	11D-2	22	Yellow	Present	Clear	6.90	326	356
	11D-3	34	Yellow	Present	Slightly turbid	6.13	219	743
Average						**6.74**	**302.0**	**471.4**

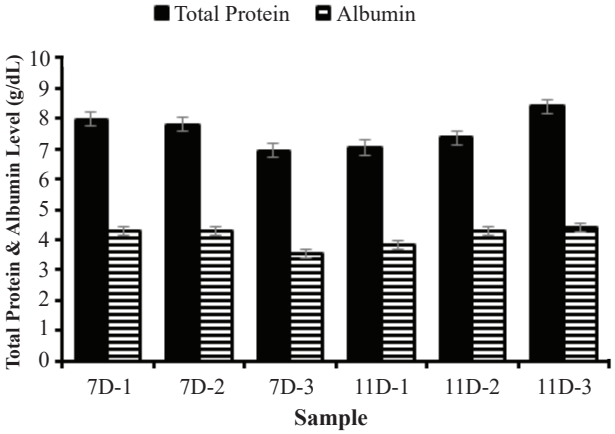

Figure 1. Total protein and albumin levels of expired PCs prior to HPL production.

Effect of HPL Treatment Towards T47D Cell Proliferation

Proliferation of T47D cells treated with 10% HPL produced from expired PCs was 1.41-1.97 times higher than 10% FBS, hence shortening the doubling time. However, high glucose levels in HPL may inhibit T47D cell proliferation (Table 2).

Discussion

In this study, the PC that was stored for more than five days still had good quality because the swirling phenomenon was still observed. This finding was similar to previous studies[13,14] which showed that swirling was still observed on the ninth day of storage. Swirling is a condition when the alignment of normal platelets causes light diffraction. When visually observed, this phenomenon appeared as a cloud-like appearance in a PC sample.[15] In addition, most of the PCs appeared to be clear, except sample 11D-3 that was slightly turbid. This condition may be caused by low pH (6.13). The colour of most PCs was yellow, except sample 7D-1 that appeared reddish-yellow in colour (Table 1). This condition may be caused by the presence of erythrocyte residues that were still left in the PC blood component (0.04 x10^6 µL; data not shown).

Proteins are macromolecules that contribute to the function and structure of the cell. Figure 1 shows that the highest protein content of HPL produced from expired PC was 8.4 g/dL (sample 11D-3). This value was lower than fresh HPL samples in our previous study. Sample B1 had the best proliferative ability with the amount of total protein =8.71 g/dL. This amount accelerated the doubling time of T47D cells to 1.07 hours.[9] Meanwhile, sample 7D-3 had the lowest total protein (6.93 g/dL). Total protein in HPL made from expired PC was not affected by platelet count (p=0.490), pH (p=0.236) or age of donor (p=0.053), but affected by glucose levels.

In this study, blood glucose level and total protein of PC prior to HPL production were negatively correlated. The p-value result of Pearson analysis was 0.041 (p<0.050). Blood protein levels are influenced by metabolic processes. Protein is broken down into amino acids through catabolism. This process can also use other energy sources, i.e. carbohydrates and fats. Carbohydrates are converted to glucose, while fats are converted to fatty acids and glycerol.[16] Acetyl CoA which is formed from glucose, fatty acids, and glycerol then converted into adenosine triphosphate (ATP).[17] If the blood glucose level is low, the metabolic process will switch to using fat or protein. Protein stored in the body

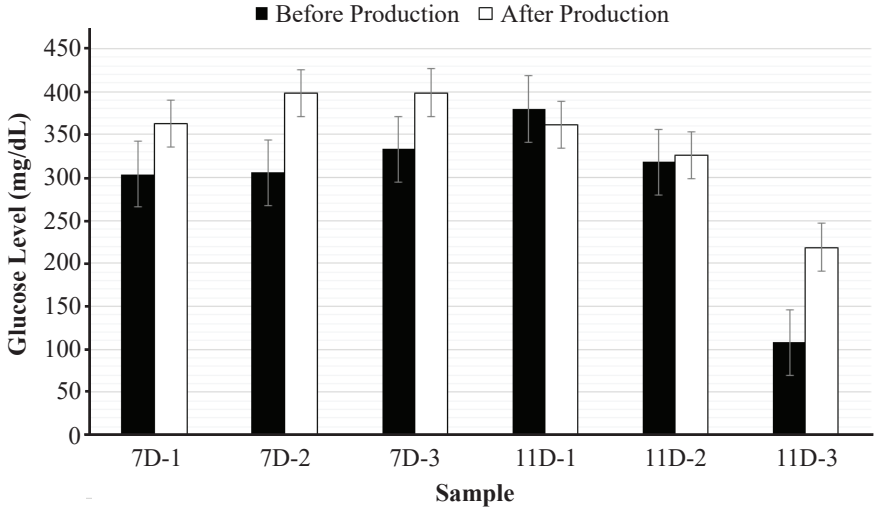

Figure 2. Glucose levels of expired PCs before and after HPL production.

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Table 2. Proliferation rate and doubling time of HPL-treated T47D cells.

Sample	Cell Proliferation (HPL 10%)			Doubling Time Analysis (HPL 5%)				
	Mean±SD	Increased Cell Proliferation (Fold)	*p* -value	Equation	Slope	R^2	Doubling Time (Hours)	*p* -value
10% FBS	132.12±1.99			y = 877.2x+2310.0	877.2	0.910	19.94	
7D-1	187.02±10.50	1.41	0.720	y = 1053.0x+761.9	1053.0	0.906	18.27	0.018**
7D-2	200.88±2.50	1.52	0.470**	y = 817.4x+2319.0	817.4	0.890	21.63	0.193
7D-3	169.24±8.44	1.28	0.255	y= 464.7x+8265.0	464.7	0.966	25.25	0.220
11D-1	221.82±31.11	1.67	0.007**	y = 908.6x+2753.0	908.6	0.920	18.98	0.002**
11D-2	229.94±38.40	1.74	0.004**	y = 910.6x+2462.0	910.6	0.894	19.26	0.000**
11D-3	262.50±65.92	1.97	0.001**	y = 900.4x+3220.0	900.4	0.926	18.64	0.000**

**LSD $p<0.001$. Proliferation rate of T47D cells treated with HPL stored for 7 and 11 days were 1.41 and 1.80 times higher than FBS, respectively.

is broken down, increasing the total protein in the blood. Total protein and albumin level prior to HPL production are shown in Figure 1.

Albumin is the most abundant protein in blood plasma produced in the liver which acts as an antioxidant.[18] Pearson correlation test between protein and albumin levels (p=0.030) revealed that albumin levels were influenced by total protein. Albumin levels rise in response to high protein levels.[9] In this study, the number of thrombocytes had no effect on total protein, but had an effect on albumin levels (p=0.046). These results are in accordance with a previous study, which explained that albumin levels in plasma correlated with platelet counts in patients with nephrotic syndrome. The lower the albumin level, the higher the platelet count.[19]

Glucose is used for cell metabolism.[20] The longer the storage duration, the lower the glucose levels in the PC.[14] Platelets require glucose as an energy source. Sample 11D-1 had the lowest platelet count ($315x10^3$ µL) and the glucose level was 380 mg/dL. Meanwhile, sample 11D-3 had the highest number of platelets ($743x10^3$ µL) and the lowest glucose level (108 mg/dL) (Table 1). High platelet count increases the amount of energy needed for cellular respiration. This condition reduced the amount of glucose in sample 11D-3 compared to 11D-1.

Wilcoxon test showed no difference in glucose levels before and after HPL production (p=0.075). In this study, an increase in glucose levels after HPL production was observed in five samples (7D-1, 7D-2, 7D-3, 11D-2, and 11D-3). This condition may be caused by glucose leakage due to thrombolytics as a result of cell membrane rupture during the freeze/thaw process. However, sample 11D-1 had lower glucose level after HPL production. Figure 2 shows the glucose levels before and after HPL production.

Cell proliferation is an extremely important stage in anticancer tests. In this study, T47D cell proliferation assay was carried out using 10% HPL. Results showed that 10% HPL increased T47D cell proliferation rate better than 10% FBS. Cell proliferation rate was significantly different in T47D cells treated with samples 7D-2, 11D-1, 11D-2, and 11D-3 compared to 10% FBS. Cell proliferation rate increased by 1.97 times when treated with 10% HPL from sample 11D-3 compared to 10% FBS. In our previous research, HPL 10% made from PC with a shelf life of 3 days increased cell proliferation 1.79 times faster than 10% FBS.[9] HPL stored for 7 and 11 days increased cell proliferation rate 1.41 and 1.80 times higher than 10% FBS, respectively. These results showed that HPL made from expired PC had the same quality as HPL made from PC with a shelf life of 3 days.

Proliferation of T47D cells was inhibited by high glucose level in sample 7D-3 (Figure 2). T47D cells treated with sample 7D-3 had the lowest proliferation rate compared to other samples, which was 1.28 times faster than FBS (Figure 3). It also had the longest doubling time (25.25 hours), which may be caused by high glucose levels (399 mg/dL) compared to other samples. High glucose levels (30 mM) did not increase the production of vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and fibroblast growth factor (FGF2) in human MSCs after 48 hours.[21] High glucose levels also inhibited bone marrow mesenchymal stem cells (BM-MSCs) proliferation by inducing cell senescence. In addition, high glucose levels activated protein kinase C (PKC), inhibited nicotinamide-

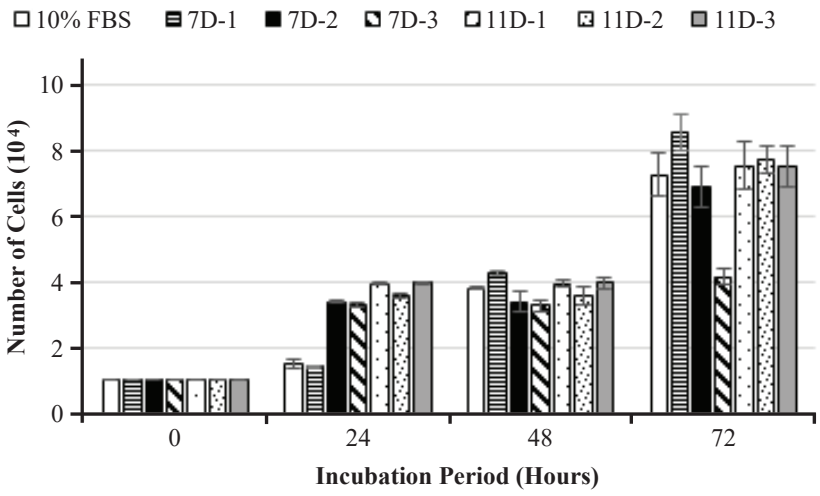

Figure 3. Glucose levels of expired PCs before and after HPL production.

adenine dinucleotide phosphate (NADPH) oxidation, and increased reactive oxygen species (ROS) production.[22] The results of Pearson correlation analysis showed that glucose levels affected cell proliferation (p=0.03). However, the relationship between these variables was negatively correlated; the higher the glucose level, the lower the T47D cell proliferation rate.

Results of cell proliferation assay using 10% HPL and doubling time analysis using 5% HPL are shown in Table 2. In the doubling time analysis, 10,000 cells/well were incubated for 72 hours. The results showed that the samples had the same cell number in the first 24 hours. The number of cells were different in each group after 48 hours of incubation. T47D cells treated with sample 7D-1 had the highest proliferation rate. Cell proliferation was also observed after 72 hours. Cells treated with sample 7D-1 had a doubling time of 18.27 hours. This time was faster than doubling time of cells cultured in FBS (19.94 hours).

Proliferation and doubling time of T47D cells that were treated with sample 7D-3 were not statistically significant when compared to 10% FBS. This may be caused by low albumin content (3.53 g/dL) in the sample (Figure 1). Albumin is an essential component in cell culture medium.[23] Albumin has been reported to initiate G1-to-S phase transition in fibroblasts.[24] In addition, high glucose level in sample 7D-3 (Figure 2) may also inhibit cell proliferation. The results of doubling time analysis are shown in Figure 3.

Higher proliferation rate in HPL-treated T47D cells may be caused by higher growth factors concentration. HPL contains chemokines that have been reported to have abilities to regulate, proliferate and differentiate MSCs.[25] HPL was also reported to slow down the formation of senescence-associated-galactosidase (SA-β-gal) in adipose-derived mesenchymal stem cells (ADMSCs). SA-β-gal is a DNA damaging enzyme that is found only in senescent cells.[26,27]

HPL did not cause any morphological abnormalities in T47D cells. Cells propagated using HPL had normal morphology compared to control cells and cells cultured in FBS. Several dead T47D cells were also observed in medium containing 10% FBS. Spherical T47D cells had lost their attachment ability to the well surface. In addition, there was also a part of the well surface that was still empty. The morphology of T47D cells grown in a medium containing 5% HPL is shown in Figure 4.

In Figure 4, cells propagated on medium containing HPL had a higher cell density compared to FBS. At 24 hours, the density of cells treated with sample 11D-2 was the highest compared to other samples. The well surface was completely filled with cells. This condition caused a decrease in cell proliferation. As a result, there was a decrease in the doubling time lower than the sample code 7D-1 at 72 hours (Figure 3). T47D cells are adherent cells. Therefore, cell proliferation will decrease when the surface of the plate is full of cells. Platelet storage lesions (PSL) are caused by long PC storage duration. Biochemical changes that may occur including a decrease in pH, the number of platelets and glucose levels.[14,28] PSL affects the quality of HPL obtained.

Conclusion

Expired PC can be used as a potential material for HPL production, since HPL produced from expired PC increases

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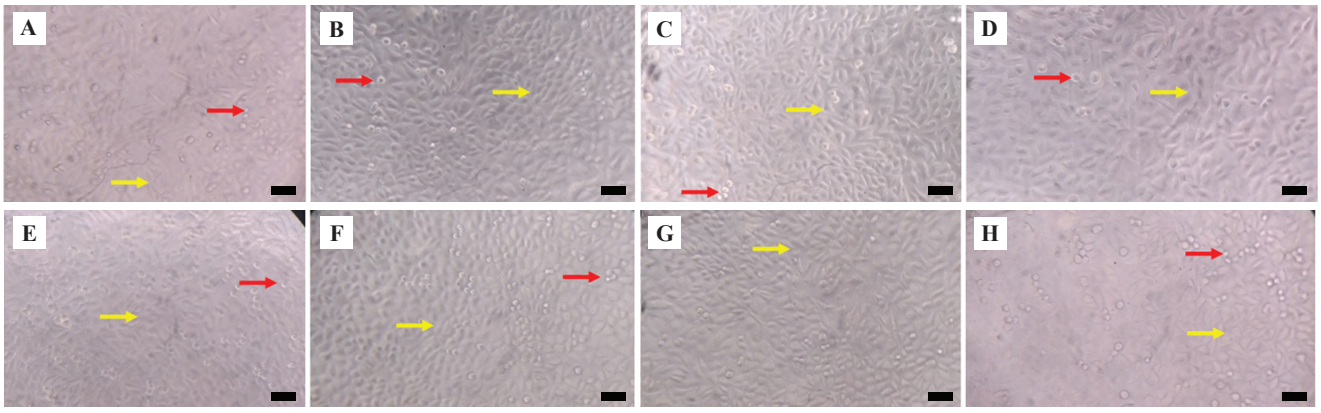

Figure 4. Morphology of T47D cells incubated for 24 hours with 5% HPL. A: control cells, B: sample 7D-1, C: sample 7D-2, D: sample 7D-3, E: 10% FBS, F: sample 11D-1, G: sample 11D-2, H: sample 11D-3. Observation was performed under an inverted microscope. Red arrows indicate dead cells, while yellow arrows indicate viable cells. Black bar: 30 µm.

cell proliferation rate and shortens cell doubling time. Expired PC has a low pH, which inhibits the glycolysis process, causing glucose levels to rise. High glucose levels in PC leads to low total protein in the plasma (blood component of PC). Cell proliferation is influenced by glucose levels, and high glucose inhibits cell proliferation.

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