

Impact of Donor Age on Human Platelet Lysate Quality and its Consequential Effects on HeLa Cell Growth in the Presence of Anti-Cancer Compounds

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Abstract

An integral aspect of anticancer experimentation involves delineating the optimal dosage of the test compound to ascertain its efficacy in targeting malignant cells. Numerous variables may influence a compound's cytotoxicity, among which is the choice of cell culture medium. Within *in vitro* settings, supplementary mediums are employed to foster cellular proliferation. Platelet lysate (PL) serves as a growth supplement, presenting an alternative to fetal bovine serum (FBS), primarily due to its incorporation of growth factors such as platelet-derived growth factor (PDGF), a component absent in FBS. The integrity of PL may be subject to various factors, including the age of the donor. This study sought to evaluate the impact of donor age on PL quality. Furthermore, it aimed to discern whether PL derived from platelet concentrate (PC) blood components of different age cohorts influences the IC_{50} value in anticancer compound assessment. Expired PCs were utilized, subsequently classified into age categories: ≤ 30 years, > 30 years, and a combination of ages. PL analysis encompassed parameters such as pH, blood profile, protein, glucose, and cholesterol levels. The investigation scrutinized the influence of PL quality, as a cellular growth supplement, on the anticancer compound cisplatin's activity against HeLa cells. Findings indicate that donor age influenced the IC_{50} value of cisplatin on HeLa cells. Notably, elevated cholesterol levels and decreased pH in PL from donor ages > 30 years were associated with reduced cisplatin toxicity.

Keywords: *cisplatin, donor age, HeLa, IC_{50} , Platelet Lysate.*

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INTRODUCTION

Cancer emerges as a significant focal point both globally and nationally. As per Globocan's, 2020 data, Indonesia harbored 213,546 individuals grappling with cancer, with cervix cancer constituting 17.2% of these cases (Global Cancer Observatory, 2020). Cervical cancer is a specific type of cancer influenced by various factors, including infection with the human papillomavirus (HPV) (Okunade, 2020). The assessment of anticancer compounds represents a crucial stage in determining the optimal dosage required to inhibit cancer cell proliferation (Kitaeva, *et al.*, 2020). Administering excessively high doses of such compounds can provoke toxicity not only in cancerous cells but also in healthy ones, thus posing the risk of harm and even mortality.

In *in vitro* studies, maintenance and accelerated cell growth often require the use of growth factor supplements. Human platelet lysate (HPL) emerges as a viable culture supplement capable of substituting fetal bovine serum (FBS) (Mentari, *et al.*, 2020; Mohamed, *et al.*, 2020). HPL production entails subjecting platelet concentrate (PC), a blood component, to iterative freeze-thaw cycles to lyse and eliminate platelet cell debris, thus releasing growth factors. PC is readily available at the Red Cross (PMI) blood bank due to its high demand. Nevertheless, PC possess a relatively brief shelf life, typically spanning five days, culminating in the disposal of expired PC. The presence of unusable PC presents an opportunity to utilize this blood component as the primary material for HPL synthesis (Dessels, *et al.*, 2016). Research indicates that HPL derived from PCs surpassing their shelf life augments T47D cell proliferation, comparable to that of non-expired PC (Mentari, *et al.*, 2022). Moreover, HPL at a concentration of 5% has exhibited a more pronounced escalation in T47D cell proliferation compared to 10% FBS, resulting in a faster doubling time (Mentari, *et al.*, 2020, 2022).

As of present, there exists no documented instance of the concurrent application of HPL along side anticancer compounds in HeLa cells. HPL boasts a rich composition of growth factors such as VEGF, IGF, and TGF, all of which assume pivotal roles in cellular proliferation, angiogenesis, and apoptosis inhibition (Durante, *et al.*, 2013; Wen, *et al.*, 2018). The utilization of HPL in cell culture closely mimics the *in vivo* conditions. By employing HPL in conjunction with anticancer compounds, it is possible to discover a more optimal dosage. In this research, the anticancer compound cisplatin will undergo *in vitro* testing on HeLa cells, with the addition of PL in the culture medium. Cisplatin stands as a widely employed therapeutic compound in cancer treatment (Dasari and Tchounwou, 2014). It functions as a neoadjuvant chemotherapy frequently utilized to reduce the size of cancerous cells before surgical intervention (Huang, *et al.*, 2021). The combination of cisplatin and paclitaxel for breast cancer treatment at Fatmawati General Hospital in Indonesia ranks 5th out of 13 other therapy methods. This therapy is used in patients aged 36–45 years and 45–55 years (Haryani, 2022), rendering it a suitable test compound for testing the impact of HPL on cell proliferation.

MATERIALS AND METHODS

The Selection of Blood Components Platelet Concentrate

The research protocol employed herein has obtained ethical clearance from the Faculty of Medicine, Gadjah Mada University (Ref. No: KE/FK/1225/EC/2023). The expired PC blood components utilized in this study were supplied by PMI Yogyakarta City. The utilized PC met the quality control standards outlined in Regulated of PERMENKES RI No. 91 in 2015 (KEMENKES 2015), as corroborated by prior investigations (Mentari, *et al.* 2020, 2022).

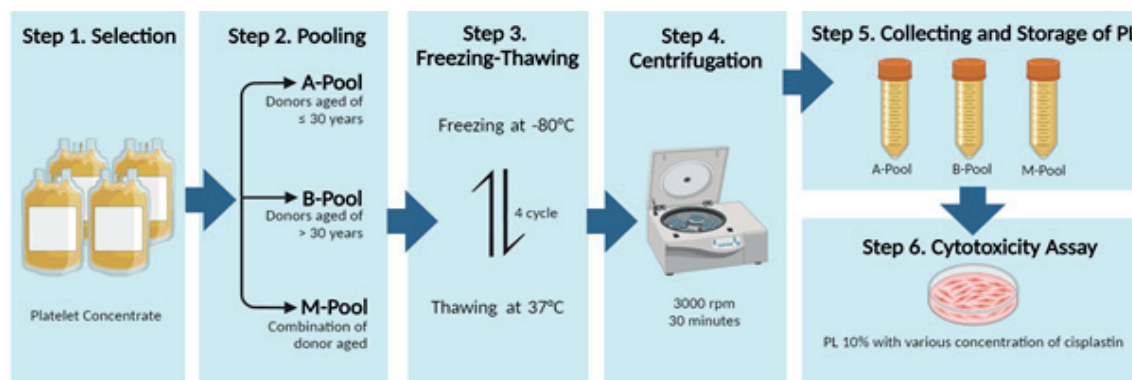


Figure 1. The pooling technique of PL production with different aged donor.

The PL was prepared using PC component pooling techniques (Figure 1). Pooling was conducted with stratification based on the age range of blood donors. The initial pooling comprised 3 bags from donors aged ≤ 30 years, while the second pooling encompassed 3 bags from donors aged > 30 years, along with 2 bags from donors with combined ages, all of whom were male with blood type O. This was due to the highest number of blood donors with type O compared to donors of other blood types.

The Human Platelet Lysate (HPL) Production

PCs that passed quality control assessments underwent four cycles of freezing and thawing. The method utilized for producing HPL was adapted from Mentari, *et al.*, (2022) Freezing was conducted at -80°C , followed by thawing at 37°C . To eliminate cellular debris, centrifugation was performed at 3000 rpm for 30 minutes. Subsequent to centrifugation, the lysate underwent measurements of glucose, cholesterol, protein levels, and hematology analysis. Glucose, protein, and cholesterol levels were determined using the colorimetric method with a KIT analyzer from Diagnostika Sistem Indonesia (DSI). Optical density measurements were taken using a UV-Vis spectrophotometer. Blood profile analysis was executed using a hematology analyzer employing the “3 diff” method.

The Use of Human Platelet Lysate (HPL) together with the Anticancer Compound Cisplatin in HeLa Cell Culture

HeLa cells were cultured in 96-well plates using RPMI 1640 medium at a density of 5000 cells/100 μl and allowed to proliferate until reaching confluence. For assessing compound toxicity, an IC_{50} value below 1000 $\mu\text{g/mL}$ is considered toxic (Prayong, *et al.* 2008). The IC_{50} analysis was conducted using cisplatin at concentrations ranging under 1000 $\mu\text{g/mL}$ (500, 250, 125, 62.5, 31.25, and 15.625 $\mu\text{g/mL}$), which were grown together with a 10% concentration of PL. The viability of cells was determined by introducing Thiazolyl Blue Tetrazolium Bromide (Merck), followed by measurement using an ELISA reader at a wavelength of 570 nm.

RESULTS

The Quality Control Process for Blood Components Platelet Concentrate (PC) as Raw Material for Making Platelet Lysate (PL)

The pooling technique represents one of the strategies used to reduce product variations in PL. In this research, pooling was conducted by categorizing PC components according to the age range of donors. Initial pooling involved donors aged ≤ 30 years, followed by subsequent pooling incorporating donors aged > 30 years and

Tabel 1. Standard of platelet concentrate blood components that will be frozen.

No	Code	Ages (years)	Swirling	pH	Thrombocyte Count ($10^3 \mu\text{l}$)	RBC ($10^3 \mu\text{l}$)
1	A1	20	Yes	6.95	740	0.13
2	A3	23	Yes	7.19	850	0.07
3	A4	29	Yes	7.27	519	0.09
4	B1	44	Yes	7.44	437	0.05
5	B2	59	Yes	7.11	729	0.05
6	B3	60	Yes	7.31	587	0.04
7	M1	23	Yes	7.23	1059	0.07
8	M2	68	Yes	7.00	766	0.06

combinations of ages. It is noteworthy that each PC bag maintained a pH >6.4, with swirling observed (KEMENKES 2015). In this study, blood type O was utilized due to its greater availability compared to other blood types. The quality control of PC components used in this research can be seen in Table 1.

Table 1 categorizes samples from donors aged ≤ 30 years as code A, those aged >30 years as code B, and those with combination ages as code M. The mean platelet count in group A was $703 \times 10^3 \mu\text{l}$, while in group B, it measured $584 \times 10^3 \mu\text{l}$, and in group M, it was $912.5 \times 10^3 \mu\text{l}$. Research has indicated a tendency for platelet counts to decrease with age (Biino, *et al.*, 2013). The frozen PC components underwent a thawing process in a water bath set at a temperature of 37°C . This freeze-thawing process was repeated four times. Results from the freeze-thawing procedure

showed that samples M1 and M2 exhibited a slight red coloration (Figure 2). Hematology analysis of samples M1 and M2 indicated residual RBC counts within the normal range, specifically $0.07 \times 10^3 \mu\text{l}$ in M1 and $0.06 \times 10^3 \mu\text{l}$ in M2 (refer to Table 1). An overview of the quality of PC after the freeze-thaw process is provided in Table 2 and Figure 2.

Following to the pooling process, the subsequent step entails centrifugation at a speed of 3000 rpm for 30 minutes. This procedure aims to eliminate clots and cellular debris formed during the freezing-thawing process. As illustrated in Figure 1, it is apparent that the sample labeled M-Pool exhibits a slightly reddish color compared to the A-Pool and B-Pool samples. Furthermore, M-Pool yields fewer cellular debris particles than the other two samples, as evidenced by the reduced presence of particles (as indicated by the white arrow).

Table 2. PC quality after the freezing-thawing process.

No	Code	Pooling	PC condition after Freezing-Thawing	pH
1	A1	A-Pool	Very clear yellow	7.7
2	A3		Yellow with turbid	
3	A4		Very clear yellow	
4	B1	B-Pool	Yellow with turbid	7.4
5	B2		Clear yellow	
6	B3		Clear yellow	
7	M1	M-Pool	Reddish yellow and slightly cloudy	7.7
8	M2		Reddish yellow and slightly cloudy	

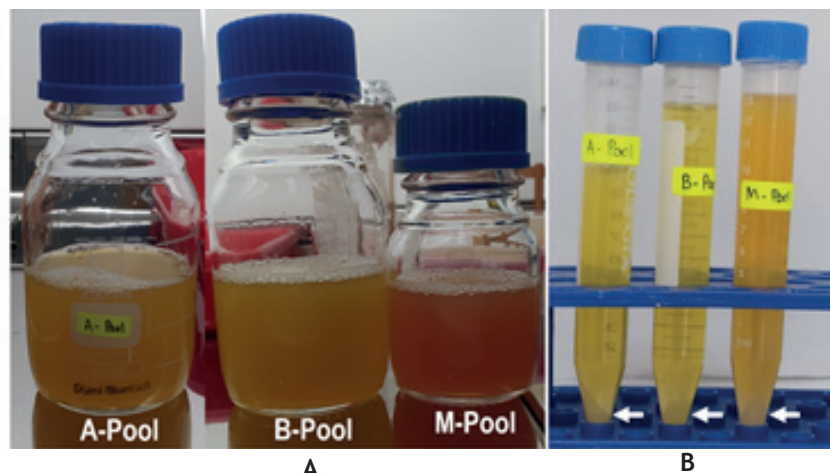


Figure 2. Pooling process of PC components that have been frozen thawing (A) and PL which has been centrifuged for 30 minutes using a speed of 3000 rpm (B).

The Total Protein in the Platelet Concentrate (PC) Blood Component and Platelet Lysate (PL)

Significant disparities were observed in the protein analysis results before and after centrifugation. The total protein content decreased subsequent to centrifugation. Specifically, samples coded B-Pool and M-Pool experienced a reduction in protein content of approximately $\pm 5\%$, while samples in the A-Pool displayed a modest increase in protein content, about 0.74%. The impact of centrifugation force on protein reduction is well-documented. Research indicates that increasing centrifugation speed from 7,000 g to 9,000 g leads

to a reduction in proteins with a molecular weight ≥ 30 kDa. Moreover, higher centrifugation speeds aid in the elimination of smaller proteins in the supernatant. Increasing the centrifugation speed from 8,000 g to 9,000 g results in the removal of larger proteins within the supernatant, particularly those with sizes of 25–150 kDa (Baskoro, *et al.*, 2017). However, during the Pair T-Test analysis, it was statistically determined that the decrease in protein content following the centrifugation process did not achieve significant difference ($p=0.246$ or $p>0.05$). The results of total protein analysis in PL before and after centrifugation are shown in Figure 3.

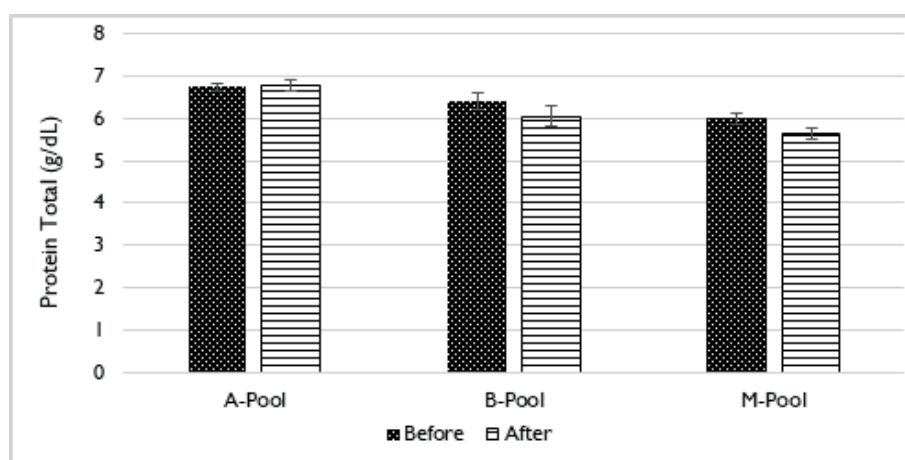


Figure 3. Total protein in Platelet Lysate (PL) before and after centrifugation did not reach a significant difference.

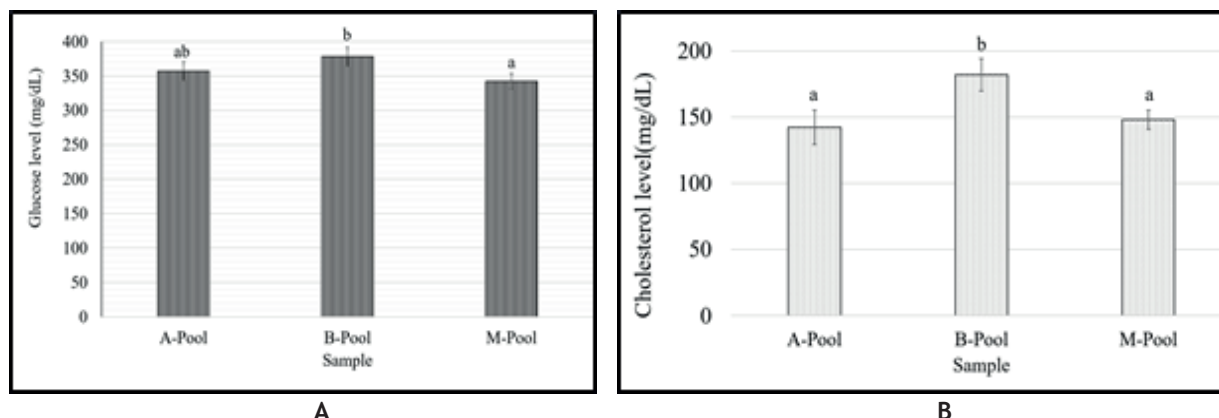


Figure 4. Glucose (A) and Cholesterol Levels (B) in each sample of Platelet Lysate (PL). Samples from donors aged ≤ 30 years are classified as code A, while those aged >30 years are designated as code B and combination ages as code M.

The Total Glucose and Cholesterol Levels in Platelet Lysate (PL) after the Centrifugation Process

The analysis results reveal that the B-Pool sample exhibits the highest glucose content compared to the other two samples, specifically 378 mg/dL. Glucose and cholesterol levels in each sample are depicted in Figure 4. The ANOVA analysis results indicated that the glucose levels in each treatment did not display significant differences ($P=0.52$ or $p>0.05$). However, in the ANOVA analysis, cholesterol levels exhibited a value of $p=0.009$ or $p<0.05$, indicating that each treatment possesses a distinct mean.

The Use of Platelet Lysate (PL) together with the Anti-Cancer Compound Cisplatin in HeLa Cell Cultures

The assessment of PL as a cell culture medium in conjunction with anticancer compounds was carried out using HeLa cells, a uterine cancer cell line. This experiment aimed to evaluate the impact of PL derived from donors across different age groups on the IC_{50} dose when utilizing the anticancer compound cisplatin. The IC_{50} experiment involved various concentrations of cisplatin, including 500, 250, 125, 62.5, 31.25, and 15.625 $\mu\text{g/mL}$. The results of the cytotoxicity test experiment revealed that PL

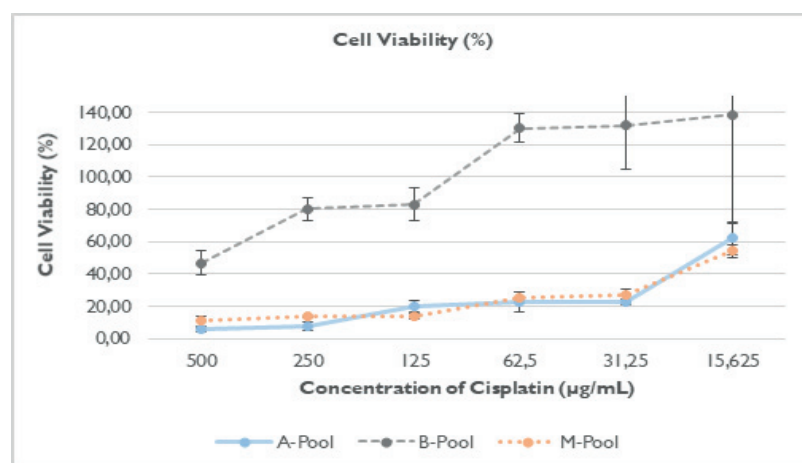


Figure 5. The percentage viability of the HeLa cell line after incubation with cisplatin at different concentrations in various supplement media (A-Pool, B-Pool, and M-Pool).

derived from donors of different ages yielded distinct IC_{50} values. The A-Pool sample (from donors aged ≤ 30 years) exhibited the highest IC_{50} value, recorded at 19.55 $\mu\text{g/mL}$. Conversely, the B-Pool sample displayed the lowest IC_{50} value at 127.15 $\mu\text{g/mL}$, while the M-Pool sample had an IC_{50} value of 19.84 $\mu\text{g/mL}$. This IC_{50} study shows the significant influence of PL quality on the efficacy of the testing process when combined with drug compounds. At the lowest concentration of cisplatin compound (15.625 $\mu\text{g/mL}$), the cells with the lowest cell viability were those from the M-Pool sample (54.09%). In contrast, the B-Pool sample showed high cell viability, measuring 138.54%. The cell viability of HeLa cells at various concentrations of cisplatin can be observed in Figure 5.

HeLa cells, are epithelial cells, tend to adhere to the surface of the growth medium as they proliferate. In the control cell sample A-Pool (A-Pool-CC), the HeLa cells appear larger in size

compared to those in B-Pool (B-Pool-CC) and M-Pool (M-Pool-CC). In the B-Pool and M-Pool samples, the cells exhibit a rounded morphology, with some displaying pigmentation (Figure 5). In this study, the inhibition of HeLa cell proliferation was influenced by the dosage of cisplatin. Higher doses resulted in decreased cell proliferation. For example, at a cisplatin concentration of 125 $\mu\text{g/mL}$ (across all samples), the cells exhibited a rounded morphology, indicative of cell death, in contrast to the 15.625 $\mu\text{g/mL}$ dose. Previous research has demonstrated the use of cisplatin on HeLa cells cultured in DMEM medium containing 10% FBS. The inhibition of cell proliferation by cisplatin is both dose-dependent and duration-dependent. At a dose of 50 $\mu\text{g/mL}$, the percentage of inhibition of cell proliferation exceeded 40-60% on day 3 (Liu, *et al.*, 2008). The morphology of HeLa cells during IC_{50} testing using the cisplatin compound in a medium containing PL supplements can be observed in Figure 6.

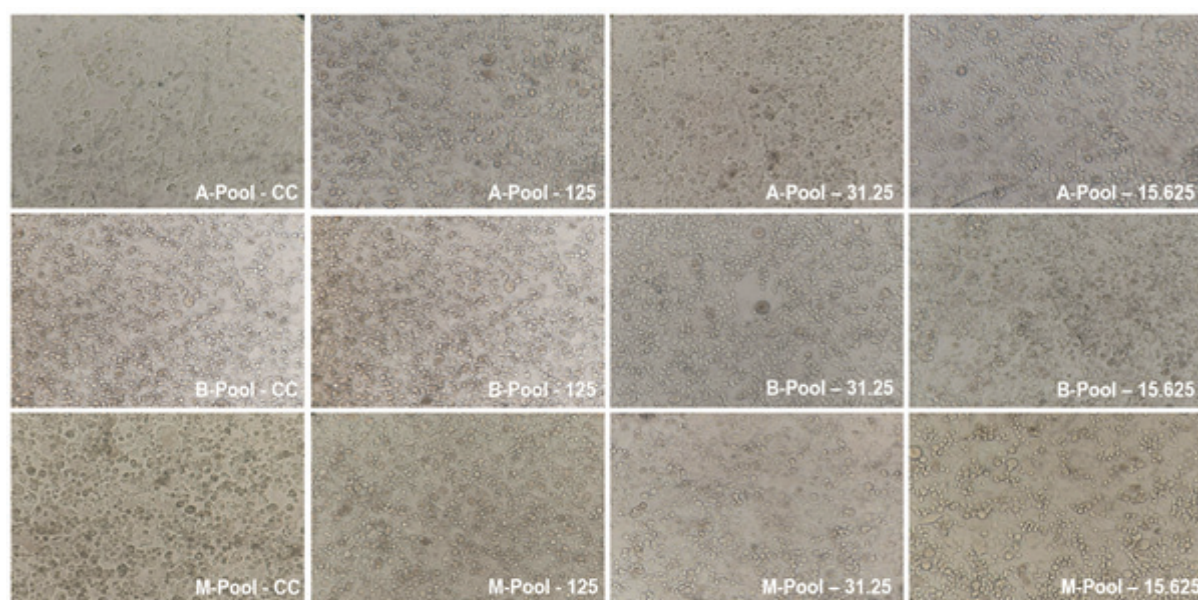


Figure 6. HeLa cell line morphology during IC_{50} analysis. The cell control code CC (Magnification 100 $\times$).

DISCUSSION

Glucose, protein, and cholesterol are essential macromolecules that significantly influence cell proliferation or death. However, in this study, the results of correlation coefficient analysis using Kendall's tau demonstrated that the levels of protein and glucose had no discernible effect on the IC_{50} value in each treatment ($p > 0.05$). High glucose levels have been shown to inhibit cell growth and extend the doubling time in T47D cell lines (Mentari, *et al.*, 2022), while being toxic to hGF normal cell lines (Mazlan, *et al.*, 2020). Additionally, elevated glucose levels do not trigger an increase in growth factors such as VEGF, HGF, or FGF2 (Weil, *et al.*, 2009). Furthermore, high glucose levels inhibit cell proliferation in BM-MSCs cultures by inducing cell senescence. Moreover, high glucose levels can induce protein kinase C (PKC) activation, inhibit NADPH oxidation and increase Reactive Oxygen Species (ROS) (Quagliaro, *et al.*, 2003).

Indeed, cholesterol demonstrates a significant influence ($p < 0.05$) on the IC_{50} value. The variance in IC_{50} values could be attributed to differences in cholesterol levels within the PL. High cholesterol levels can affect the cellular uptake of compounds, as cholesterol possesses the capability to diffuse within the cell membrane, consequently reducing cell membrane permeability. Notably, the B-Pool sample, characterized by higher cholesterol levels, showed a correspondingly lower IC_{50} value. This relationship is shown in Figure 3, where B-Pool samples with age variations exceeding 30 years (ranging between 44-60 years) showed the highest cholesterol levels compared to the other two samples. Despite this, the average total cholesterol measured at 182 mg/dL remains within normal limits. The M-Pool sample recorded the second-highest cholesterol level at 148 mg/dL, while the A-Pool sample showcased the lowest at 142.5 mg/dL. Cholesterol serves as a vital

component for constructing cell membrane structures (Subczynski, *et al.*, 2017). The levels of cholesterol in the bloodstream are influenced by various factors, including age. Studies have indicated that cholesterol levels tend to be higher in individuals aged 41-50 years (Mentari, *et al.*, 2019), a finding consistent with research conducted by Soleha (2012), which also reported elevated cholesterol levels in the age groups of 40-49 years and 50-59. In Figure 4, it is evident that the B-Pool samples, with age variations exceeding 30 years (ranging between 44-60 years), showed the highest cholesterol levels compared to the other two samples. The average total cholesterol was measured at 182 mg/dL, falling within the normal range. The second-highest cholesterol level was observed in the M-Pool sample (148 mg/dL), with the A-Pool sample demonstrating the lowest level (142.5 mg/dL). Cholesterol plays a critical role in maintaining cell membrane permeability. Higher cholesterol levels correlate with lower cell membrane permeability (Blosser, *et al.*, 2020). Thus, to explore whether cholesterol influences the uptake of compounds into cells, PL testing is concurrently conducted alongside testing for anticancer compounds (cisplatin). Cisplatin stands as a widely used anticancer medication, frequently utilized as a positive control in *in vitro* assessments of anticancer compounds. Its mechanism involves binding to DNA within cancer cells, thereby causing disruptions in replication and transcription processes that ultimately culminate in cell death via apoptosis (Liu, *et al.*, 2008). The induced DNA damage triggers the activation of the P53 gene, resulting cell cycle arrest (Dasari and Tchounwou, 2014). Prior studies have examined the effects of phospholipids on HeLa cells, and differences in the cell cycle profiles of HeLa cells when cultured with PL versus FBS have been documented. Most cells underwent the S phase in both FBS and PL cultures, a higher percentage of cells entered the S phase (approximately 50%) when exposed to FBS

compared to PL (approximately 45%). In contrast, the proportion of cells in the G0/G1 phase was greater (approximately 40%) in PL cultures compared to FBS (approximately 35%)(Fazzina, *et al.* 2016).

The pH value plays a crucial role in influencing drug uptake into the cell membrane. In this study, the B-Pool sample showed a pH of 7.4, lower than that of the A-Pool and M-Pool sample, which recorded a pH of 7.7. A reduction in pH levels induces alterations in cell membrane permeability due to drug protonation. Interestingly, changes in pH towards acidity have been observed to diminish the toxicity of Doxorubicin, as demonstrated in testing on HeLa and A498 cells (kidney cell line) (Trebinska-Stryjewska, *et al.*, 2020).

CONCLUSION

The age of the donor influences the quality of the PL produced. Donors aged >30 years have higher cholesterol levels and low pH than those aged ≤30 years and a combination of ages. High cholesterol and low pH in PL are associated with reduced the IC₅₀ values.

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