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3D Culture Cells Technique for Exosomes Isolation of HEK293 and its Application on WiDr Cells

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ABSTRACT

Three-dimensional (3D) culture is a technique commonly utilized in bioprocessing and biomedical research. Exosomes have been investigated as carriers for medications in numerous studies employing 3D culture methodologies. The objective of this research is to employ 3D cell culture for the isolation and treatment of exosomes targeting colon cancer cells. The isolation of exosomes obtained from HEK293 cells was conducted through the ultracentrifugation technique. Subsequently, exosome treatment was administered to WiDr cells at concentrations of 3.5 µg/ml, 7 µg/ml, and 14 µg/ml.The validation of molecular markers of exosomes (*CD9* and *CD81*), along with *BAX, BCL-2*, and *CD133*, was performed using qRT-PCR. The findings revealed the successful isolation of exosomes derived from HEK293 cells, which exhibited the expression of markers *CD9* and *CD81*. Furthermore, the expression of *BAX* and *BCL-2* indicated the potential of exosomes to induce apoptosis, while the expression level of *CD133* decreased with treatment at varying concentrations. These results suggest that exosome treatment has the capability to impede the proliferation of WiDr cells and reduce the expression of *CD133*, thereby signifying the potential application of exosomes as an *in-vitro* model for investigating cancer therapy in the future.

1. Introduction

Colon cancer, also known as colorectal cancer, is a type of adenocarcinoma that originates from the glandular epithelial cells found in the large intestine, specifically the rectum. The development of colon cancer occurs as a result of acquired epigenetic mutations within these cells (Alzahrani *et al.* 2021). Colon cancer occurs in more than 931,950 cases worldwide, and 935,173 people die yearly due to colon cancer. In Indonesia, colon cancer that occurs in men is the second ranks after lung cancer, with a percentage of 11.9% or 21,764 of the total cancer cases (Sutnick and Gunawan 2020). Oncologists often perform colon cancer treatments, including surgery, chemotherapy and radiotherapy (Rai *et al.* 2023). Technological developments in modern medicine have led to regenerative approaches to treating cancer, exosome therapy stands out as a promising approach in the fight against cancer.

Exosomes are extracellular vesicles measuring 40-100 nm secreted by all cells, including cancer cells. Exosomes may be helpful for therapeutic since they include a variety of proteins, RNA, and DNA from the original cell (Bellavia *et al.* 2017). Exosomes play a crucial role in a variety of pathological conditions, including cancer (Kar *et al.* 2023), liver and kidney disease, neurodegenerative disorders, and various cardiopulmonary disorders (Ailawadi *et al.* 2015). Exosomes possess inherent cargo-carrying capabilities, rendering them highly potential therapeutic agents for a wide range of ailments, such as cancer and degenerative diseases. These microscopic vesicles can be extracted from various cell sources, including Human Embryonic Kidney cells (HEK293).

The HEK293 cell line, frequently utilized in the production of therapeutic proteins, has also been employed in the generation of exosomes for

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therapeutic purposes, alongside stem cells and immune cells (Hu *et al.* 2018) (Malm *et al.* 2020). HEK293 cells, possessing stable transfection capabilities, are considered as normal cells that are highly suitable for both exosome production and modification, as highlighted by Wang *et al.* in 2023. HEK293 cells express E1A and E1B, which allows continuous culture The apoptosis of HEK293 cells can be hindered and the transcription pathways as well as cell cycle control can be disrupted through various means (Berk 2005). The research employs apoptosis markers *BAX* and *BCL-2*, both belonging to the *BCL-2* gene family. *BCL-2* functions as an anti-apoptotic factor, whereas *BAX* acts as a pro-apoptotic factor (Putra *et al.* 2015). The exosomes containing miRNA-129-5p have been shown to trigger apoptosis in colon cancer cells, while also suppressing their proliferation, as demonstrated by Ji *et al.* in 2021.

A three-dimensional (3D) cell culture refers to a synthetically engineered setting where biological cells are cultivated or engage with their environment across all three spatial dimensions. In contrast to two-dimensional (2D) environments, three-dimensional (3D) cell cultures provide a conducive setting for cell growth all directions *in-vitro*, simulating their in-vivo characteristics (Malhão *et al.* 2022). *In-vitro* 3D cell spheroids have proven to be comparable to *in-vivo* studies for investigating the toxicity of drug compounds, and resulting in a higher cytotoxic effect than in 2D culture (Prasetyaningrum and Septisetyani 2019), Furthermore, it serves as a noteworthy therapeutic stimulus that can be examined on experimental animals to assess the safety of drugs prior to conducting clinical trials (Costard *et al.* 2021).

Isolate exosomes from HEK293 cells in 3-dimensional culture, and exosome treatment is carried out on WiDr cells with 3-dimensional cell culture to see cell apoptosis (*BAX* and *BCL-2*) and growth of *CD133*+ WiDr cells. The WiDr cell line is a cancer cell isolated from a woman's colon with colorectal cancer.

2. Materials and Methods

2.1. 3D culture HEK293

HEK293 cells (ATCCCRL 1573) were cultured in 6-well Ultralow Attachment Surfaces plates (Merck KGaA, Darmstadt, Germany), with 50,000 cells seeded per well in DMEM medium (Dulbecco's Modified Eagle's Medium) (Merck KGaA) supplemented with 10% Fetal Bovine Serum (FBS) (Merck KGaA), 100 units/L penicillin, and 100 g/ml streptomycin. The cells were then placed in an incubator at 37°C with 5% CO_2. Once multicellular spheres had formed, the media was harvested and transferred to a 50 ml tube, followed by centrifugation at 750 × g for 5 minutes. The resulting supernatant containing HEK293 was collected for exosome isolation.

2.2. Isolation Exosomes Derived HEK293 Cell

The process of isolating exosomes derived from HEK293 cells in this investigation involved the utilization of ultracentrifugation. Initially, the cell supernatant was subjected to centrifugation at a force of 2,000 × g for a duration of 20 minutes at a temperature of 4°C. Subsequently, the supernatant obtained from the HEK293 cells was carefully transferred into a centrifuge tube for ultracentrifugation using a W32Ti rotor (L-80XP; Beckman Coulter, Brea, CA, USA) at a force of 10,000 × g for a period of 30 minutes at 4°C. This step facilitated the isolation of exosomes, which were then transferred into a fresh tube and subjected to further centrifugation at a force of 100,000 × g for a duration of 70 minutes at 4°C. The resulting pellet, containing the exosomes, was subsequently resuspended in PBS to eliminate any debris protein originating from the cells. Following this, the resuspended exosomes were subjected to another round of centrifugation at a force of 100,000 × g for a period of 60 minutes at 4°C. Ultimately, the pellets obtained contained the exosomes, which were resuspended in 100 µL of PBS. To validate the obtained exosomes, molecular techniques such as quantitative PCR were employed, specifically targeting *CD9* (236 bp) and *CD81* (246 bp).

2.3. WiDr Treatment by Exosomes

WiDr (ATCC CCL 218) cells were plated at a density of 50,000 cells per well in RPMI 1640 medium (Roswell Park Memorial Institute) (Merck KGaA) using 6-well Ultralow Attachment Surfaces plates. The cells were then incubated at 37°C with 5% CO_2 for 18-20 hours to allow for sphere growth. Subsequently, exosomes derived from HEK293 cells were added to the WiDr cells at concentrations of 3.5 µg/ml, 7 µg/ml, and 14 µg/ml. The culture was maintained for an additional 48 hours with 5% CO_2 (Faruqu *et al.* 2018). Following incubation, the cells were harvested, processed into dry pellets, and subjected to qRT-PCR analysis to assess

the expression levels of apoptotic markers *BAX* and *BCL-2*, as well as the cancer stem cell marker *CD133* (180 bp).

2.4. WiDr Cell Morphology and Cell Viability

HEK293 cells and WiDr cells were observed from the 6 wells Ultralow Attachment Surfaces plates under an inverted phase-contrast microscope. The photograph of WiDr cells were captured using an inverted microscope connected to a computer using The software used for capturing images, Dinocapture 2.0, was employed in conjunction with the Dino-lite-Digital Microscope from the Netherlands, Europe. To determine cell viability, both live and dead cells were quantified using a haemocytometer and trypan blue staining. The resulting values were then calculated as a percentage of viability. This dataset represents the average of four separate experiments, with the standard deviation (SD) also taken into account.

2.5. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

The RNeasy kits from Qiagen, Hilden, Germany were utilized to extract mRNA from cells, while the SuperScript III Reverse Transcriptase from Invitrogen, Carlsbad, CA was used for reverse transcription, following the manufacturer's guidelines. The quantitative reverse transcription polymerase chain reaction (qRT-PCR) technique was employed to evaluate the expression levels of *BAX, BCL-2, CD9, CD81*, and *CD133* markers. The primer sequences used in this study are provided in Table 1. PCR mix reactions were carried out using SsoFast EvaGreen Supermix from BioRad, Hercules, CA on the Icycler iQ5 machine from BioRad. The thermocycler conditions consisted of an initial denaturation at 95°C for 5 seconds, followed by 40 cycles of denaturation at 98°C for 5 seconds and annealing–elongation at the optimal primer annealing temperature (Table 1) for 10 seconds. The expression levels were normalized to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) reference gene. The relative RNA expression levels were determined using the comparative Ct (ΔΔCt) method (Rao *et al.* 2013).

2.6. Data analysis

Cell morphology data were analyzed descriptively. Log transformation was used to normalise the data. The analysis was carried out using One way ANOVA (Microsoft excel 2019) tests all comparisons were conducted using a significance level of 0.05, which was deemed to be statistically significant. The findings are displayed as mean ± standard deviation.

3. Results

3.1. Exosome Derived from HEK293

HEK293 (Human Embryonic Kidney) cells are cell lines derived from human embryonic kidney cells with epithelial morphological characteristics. HEK293 cells have been successfully cultured in 3D technique using the 6 wells Ultra Low Attachment Surfaces Plate formed multicellular spheroids, the morphology of the sphere transformed from round to oblong, with a size ranging from 40 to 100 µm. (Figure 1). The cell pellets containing exosomes were successfully isolated using the ultracentrifugation method in 3D cell culture. The concentration obtained was 1.47 µg/ml, which was derived from a supernatant volume of 32 ml. Validation of exosome obtained was carried out using qRT-PCR for the expression of *CD9* and *CD81*, the mRNA level expression shown

Table 1. Primer sequences used for qRT-PCR

Primer		Nucleotide sequences (5'-3')	Annealing (°C)	References
CD9	F	TCTTGGTGATATTCGCCATT	48	(Kumar *et al.* 2015)
	R	TTCGAGTACGTCCTTCTTGG		
CD81	F	CTGTATCTGGAGCTGGGAGA	53	(Kumar *et al.* 2015)
	R	GAACTGCTTCACATCCTTGG		
BCL-2	F	CCCGAGAGGTCTTTTTCCGAG	56	(Hussain *et al.* 2015)
	R	CCAGCCCATGATGGTTCTGAT		
BAX	F	GCTCTAAAATCCATCCAG	48	(Hussain *et al.* 2015)
	R	CCTCTCCATCATCAACTT		
CD133	F	TCTTGACCGACTGAGACCCAAC	53	(Tirino *et al.* 2011)
	R	ACTTGATGGATGCACCAAGCAC		
GAPDH	F	CGGATTTGGTCGTATTGG	56	(Tian *et al.* 2010)
	R	TCAAAGGTGGAGGAGTGG		

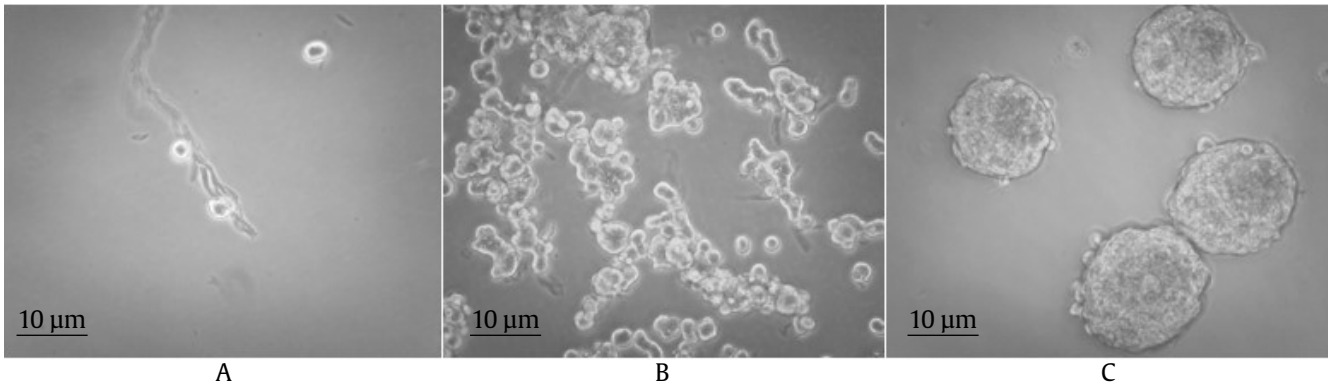

Figure 1. HEK293 cell culture successfully grown using 3D culture. (A) D0, the cell population was in the single form, not attached to the substrate, (B) D3, the cells attached each other to form spheres, (C) D7, the cells showed multicellular spheroids form

in Figure 2. The results of level expression indicated that the exosome were obtained in the cell pellet of supernatant of HEK293.

3.2. Exosome Treatment on WiDr Cells

The morphology of WiDr cells treated by exosomes are presented at Figure 3. The sphere morphology of WiDr cells without exosome showed round, 40-100 µm in size, while the sphere in exosome treated showed oblong until occurred cell shrinkage, 20-60 µm in size, the cells population were not demonstrating multiseluler sphere formed.

3.3. Viability of Widr Cells after Exosome Treatment

The viability percentage of WiDr cells after being treated with exosomes presented in Figure 4. The cells viability showed different percentage on its concentration of exosome treated. The WiDr cells were treated 3.5 µg/ml, 7 µg/ml, and 14 µg/ml exosomes resulting in a calculated average cell viability of 73.44% with a standard deviation of 8.74%. Two concentrations showed the percentage above 75%, indicating that there were more live cells observed than dead cells in this treatment. WiDr cells showed low percentage of viability 63.35% at concentration 14 µg/ml, which indicated the number of dead cells was higher than exosome concentration 3.5 µg/ml and 7 µg/ml.

3.4. Expression of Molecular Marker *BAX* and *BCL-2*

To further assess the effect of HEK293 exosomes on WiDr cell apoptosis, we evaluated the manifestation of proteins associated with apoptosis (*BAX* and *BCL-2*). The qRT-PCR results showed that *BCL-2* expression

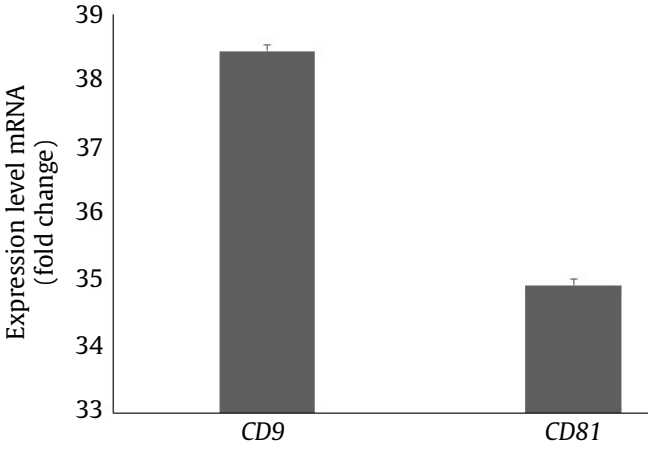

Figure 2. mRNA expression level of markers exosomes *CD9* and *CD81* are indicated with a different asterisk

at each treatment concentration decreased, and *BAX* expression increased at concentrations of 3.5 µg/ml and 14 µg/ml. Figure 5 revealed that exosomes the apoptosis rate of WiDr cells was markedly reduced by exosomes from HEK293 cells. These findings indicate a potential crucial involvement of HEK293 cell-derived exosomes in promoting apoptosis in WiDr cells.

3.5. Expression of Molecular Marker *CD133*

The expression of cancer stem cell in this study were carried out using the *CD133* marker gene, which was performed using the qRT-PCR technique presented in Figure 6. Expression levels of the *CD133* cancer stem cell marker gene in WiDr cells treated by exosomes 3.5 µg/ml, 7 µg/ ml, and 14 µg/ml resulted lower than the negative control. This research is also equipped with a schematic of an experimental study (Figure 7).

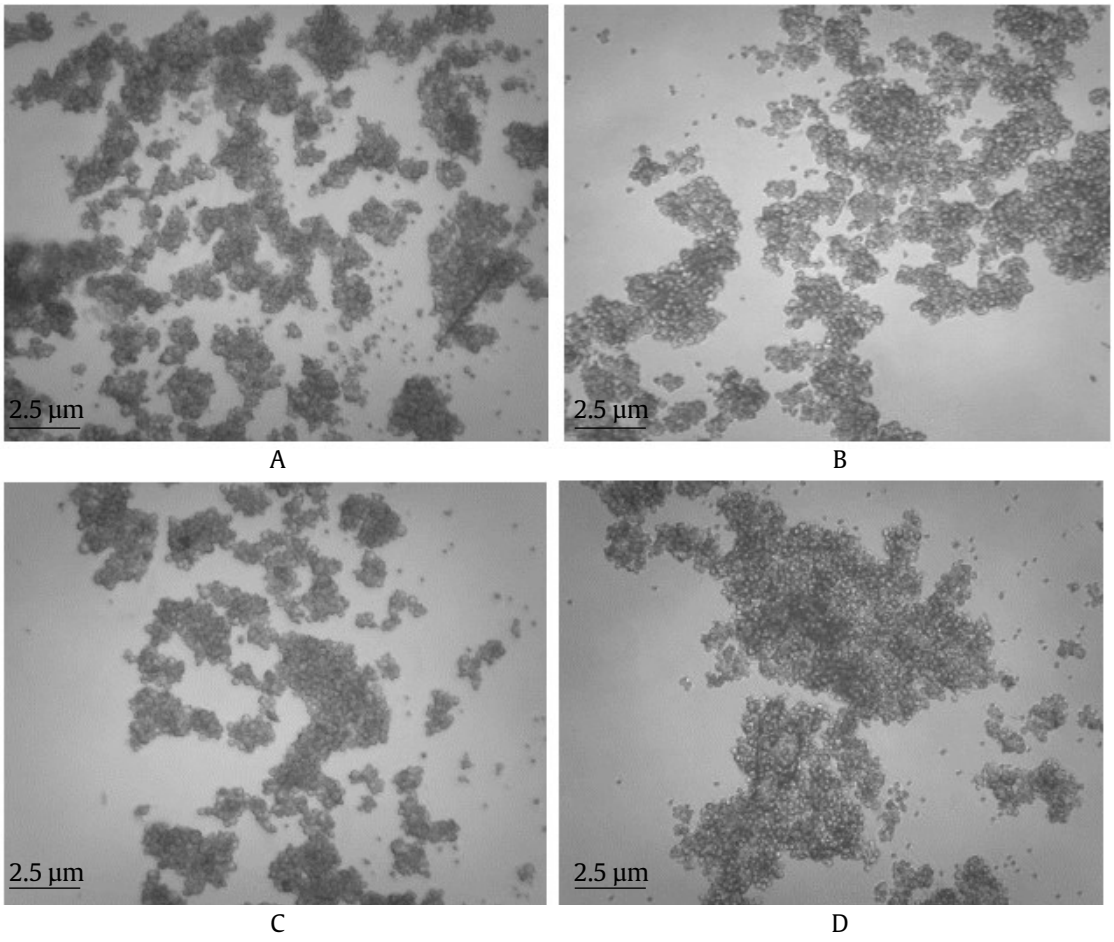

Figure 3. WiDr Colon Cancer Cells with exosome treatment. (A) 3.5 µg/ml, (B) 7 µg/ml, (C) 14 µg/ml, and (D) WiDr cells without the addition of exosomes. The morphology showed shrinking cell size and appearing denser, the cells population did not formed multicellular sphere

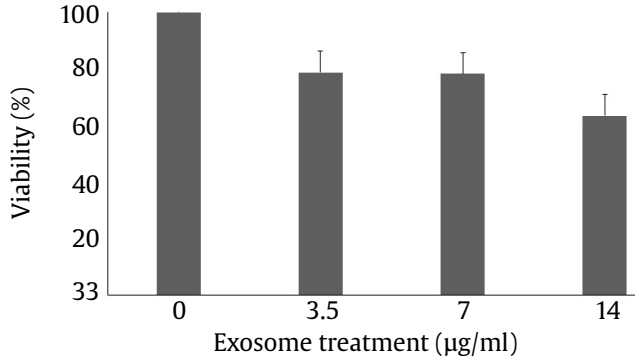

Figure 4. Percentage of WiDr cell viability after treatment of exosomes cultures incubated for 48 hours. Cells treated with exosomes showed decreased cell viability compared to cells control without treatment

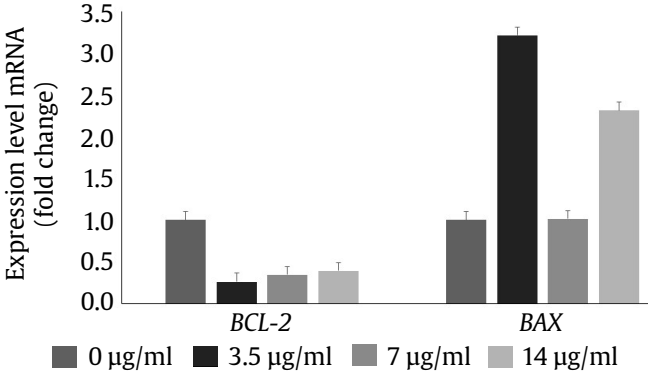

Figure 5. Graph of *BCL-2* and *BAX* gene expression levels in WiDr cell samples treated with exosomes. WiDr cells treated with exosomes at all concentrations *BCL-2* gene was lower than the untreated cells. The expression level of *BAX* protein in the respective treatments was 3.5 µg/ml and 14 µg/ml higher than the untreated cells

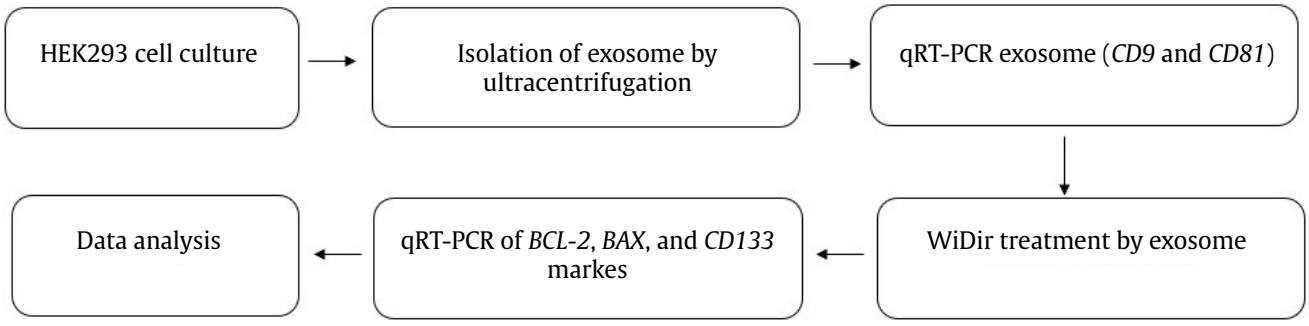

Figure 7. Schematic of the experimental study

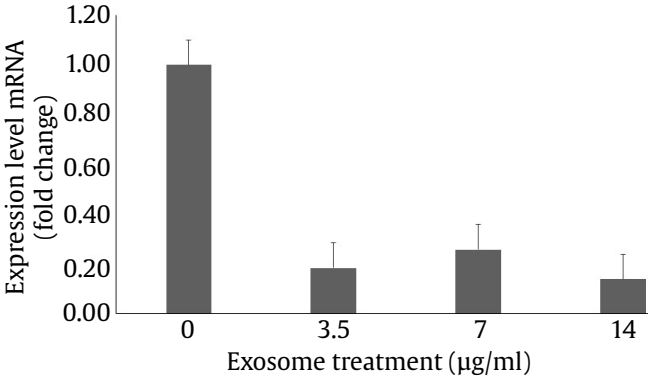

Figure 6. CD133 gene expression levels in WiDr cell samples treated with exosomes. All treatment concentrations showed lower of CD133 gene expression than the untreated cells.

4. Discussion

Exosomes exhibit promise in the realm of cancer treatment for their ability to offer therapeutic solutions owing to their minimal immunogenic response. These vesicles, released through cell exocytosis, are well-tolerated by a wide range of cell types, making them suitable for the development of innovative biomanufacturing systems aimed at drug administration and cancer treatment (Bagheri *et al.* 2020). 3D cell culture allows enhanced communication between cells by enabling spatial cell-cell interactions (Edmondson *et al.* 2014), it creates an optimal setting for exosome production and release. Throughout this research, exosome isolation was taken from HEK293 cell supernatant grown in 3D cell culture. Exosomes are microscopic membrane vesicles found in the supernatants of cell cultures (Logozzi *et al.* 2020). The HEK293 cell line, while lacking tumorigenic or toxic properties towards other cells both *in vitro* and *in vivo*, presents uncertainties regarding the intrinsic effects of exosomes derived from naïve HEK293 cells. Therefore, comprehensive investigations are necessary to elucidate the characteristics of HEK293-derived exosomes (Kim *et al.* 2021; Zhang *et al.* 2023).

Exosomes were successfully isolated using the ultracentrifugation method in 3D cell culture. The ultracentrifugation method is commonly used for isolating exosomes and producing sufficiently pure exosomes (Panlilio 2020). Exosomes contain annexins and the proteins tetraspanin (*CD9* and *CD81*), which act as exosome markers. Expression levels in exosome marker assays indicate that 3D culture conditions and exosome isolation methods are suitable for *in-vitro* exosome studies. The production of exosomes is significantly higher in 3D spheroid culture compared to 2D culture, potentially due to the non-adherent round cell morphology inherent in the former (Kim *et al.* 2018).

The treatment of exosomes in this study had an effect on the morphology of WiDr. In this study, WiDr cells were observed not to form spheroids, which was possible due to several factors, Specifically due to exosome therapy, there was a reduction in the quantity of cells and a decline in cell survival (Sirenko *et al.* 2015). The shrink cells of WiDr cells is one of the positive effects of adding exosomes as a cancer therapy agent (Zhang *et al.* 2006). In recent years, there has been extensive utilization of preclinical approaches involving exosomes derived from HEK293 cells. *In-vivo* breast cancer development is inhibited by HEK293-derived exosomes that contain let-7a miRNA (Morishita *et al.* 2017). Katakowski *et al.* (2013) In this investigation, WiDr cells were exposed to exosomes derived from HEK293 cells, leading to the examination of apoptosis markers and *CD133* expression in WiDr cells in an *in-vitro* setting. Although this study has limitations because the role of HEK293 exosomes was only evaluated *in-vitro*, this is the latest study to show that HEK293-derived exosomes without drug insertion can induce apoptosis

and decrease *CD133* expression of WiDr cells *in-vitro* with 3D cell culture. Exposure to radiation resulted in elevated levels of *BAX* protein and reduced levels of *BCL-2* protein in exosomes derived from HEK293 cells, which was induced by exosomal miR-22 and led to heightened apoptosis (Konishi *et al.* 2020). Exosomes encompass a diverse array of molecules, comprising proteins, nucleic acids, and lipids. The composition of exosomes' cargo can differ significantly, and certain proteins in exosomes may have different effects on *BAX* gene expression. In this study, it is possible that at a concentration of 7 µg/ml, certain proteins in exosomes contributed to the downregulation of *BAX*. Exosomes derived from HEK293 cells encompass miRNAs that play a dual role in regulating cellular functions. These miRNAs can either facilitate the preservation of normal cellular processes or exert their influence as either tumor suppressors or promoters, contingent upon the specific target gene they interact with. Zhou *et al.* (2018) discovered that, consistent with prior research, the exosomes derived from HEK293 cells exhibited the ability to trigger apoptosis. This was achieved through the downregulation of *BCL-2* expression and the upregulation of *BAX* expression.

Cancer therapy using exosomes has been further developed. *CD133*, also known as *AC133* or prominin-1, is a glycoprotein located on the cell membrane. It serves as a surface marker for cancer stem cells (CSCs) in different cancer types, including colon, liver, and lung cancer. In the case of colon cancer, patients with increased *CD133* expression levels are at a higher risk of developing metastasis and experiencing relapse (Sakaue *et al.* 2019). In this study, exosomes from HEK293 cells with all treatment concentrations given to WiDr cells expressed a decrease in *CD133. CD133* expression was higher at a concentration of 7 µg/ml due to the influence of the dose-dependent (Copaescu *et al.* 2021) and several other influencing factors, such as cell response to stress and microenvironment (Wan *et al.* 2020).

Based on research that has been conducted and increasing research in this area, Extracellular vesicles such as exosomes will identify the mechanisms by which these exosome molecules interact under three-dimensional (3D) various factors that can lead to the onset of diseases may uncover novel opportunities for the creation of exosome biomarkers that hold significance in the detection, prediction, and treatment of cancer.

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References

Ailawadi, S., Wang, X., Gu, H., Fan, G.C., 2015. Pathologic function and therapeutic potential of exosomes in cardiovascular disease. *Biochim Biophys Acta-Mol Basis Dis*. 1852, 1–11. DOI:10.1016/j.bbadis.2014.10.008

Alzahrani, S.M., Al Doghaither, H.A., Al-Ghafar, A.B., 2021. General insight into cancer: an overview of colorectal cancer (review). *Mol Clin Oncol.* 15, 271. DOI:10.3892/MCO.2021.2433

Bagheri, E., Abnous, K., Amel, S., Mohammad, S., 2020. Targeted doxorubicin-loaded mesenchymal stem cells-derived exosomes as a versatile platform for fighting against colorectal cancer. *Life Sci*. 261, 118369. DOI:10.1016/j.lfs.2020.118369

Bellavia, D., Raimondo, S., Calabrese, G., Forte, S., Cristaldi, M., Patinella, A., Memeo, L., Manno, M., Raccosta, S., Diana, P., Cirrincione, G., Giavaresi, G., Monteleone, F., Fontana, S., De Leo, G., Alessandro, R., 2017. Interleukin 3- receptor targeted exosomes inhibit *in vitro* and *in vivo* chronic myelogenous Leukemia cell growth. *Theranostics*. 7, 1333–1345. DOI:10.7150/thno.17092

Berk, A.J., 2005. Recent lessons in gene expression, cell cycle control, and cell biology from adenovirus. *Oncogene.* 24, 7673–7685. DOI:10.1038/sj.onc.1209040

Copaescu, A., Choshi, P., Pedretti, S., Mouhtouris, E., Peter, J., Trubiano, J.A., 2021. Dose dependent antimicrobial cellular cytotoxicity—implications for *ex vivo* diagnostics. *Front Pharmacol.* 12, 1–7. DOI:10.3389/fphar.2021.640012

Costard, L.S., Hosn, R.R., Ramanayake, H., O'Brien, F.J., Curtin, C.M., 2021. Influences of the 3D microenvironment on cancer cell behaviour and treatment responsiveness: A recent update on lung, breast and prostate cancer models. *Acta Biomater.* 132 , 360–378. DOI:10.1016/j.actbio.2021.01.023

Edmondson, R., Broglie, J.J., Adcock, A.F., Yang, L., 2014. Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors. *Assay Drug Dev Technol.* 12, 207–218. DOI:10.1089/adt.2014.573

Faruqu, F.N., Xu, L., Al-Jamal, K.T., 2018. Preparation of exosomes for siRNA delivery to cancer cells. *J Vis Exp*. 142, e58814. DOI:10.3791/58814

Hu, J., Han, J., Li, H., Zhang, X., Liu, L.L., Chen, F., Zeng, B., 2018. Human embryonic kidney 293 cells: a vehicle for biopharmaceutical manufacturing, structural biology, and electrophysiology. *Cells Tissues Organs.* 205, 1–8. DOI:10.1159/000485501

Hussain, H., Al-Harrasi, A., Al-Rawahi, A., Green, I.R., Csuk, R., Ahmed, I., Shah, A., Abbas, G., Rehman, N.U., Ullah, R., 2015. A fruitful decade from 2005 to 2014 for anthraquinone patents. *Expert Opin Ther Pat.* 25, 1053–1064. DOI:10.1517/13543776.2015.1050793

Ji, Y., Ji ,J,. Yin, H., Chen, X., Zhao, P., Lu, H., Wang, T., 2021. Exosomes derived from microRNA-129-5p-modified tumor cells selectively enhanced suppressive effect in malignant behaviors of homologous colon cancer cells. *Bioengineered.* 12, 12148–12156. DOI:10.1080/21655979.2021.2004981

Kar, R., Dhar, R., Mukherjee, S., Nag, S., Gorai, S., Mukerjee, N., Mukherjee, D., Vatsa, R., Chandrakanth Jadhav, M., Ghosh, A., Devi A., Krishnan, A., Thorat, N.D., 2023. Exosome-based smart drug delivery tool for cancer theranostics. *ACS Biomater Sci Eng.* 9, 577–594. DOI:10.1021/acsbiomaterials.2c01329

Katakowski, M., Buller, B., Zheng, X., Lu, Y., Rogers, T., Osobamiro, O., Shu, W., Jiang, F., Chopp, M., 2013. Exosomes from marrow stromal cells expressing miR-146b inhibit glioma growth. *Cancer Lett*. 335, 201–204. DOI:10.1016/j.canlet.2013.02.019

Kim, M., Yun, H.W., Park, D.Y., Choi, B.H., Min, B.H., 2018. Three-dimensional spheroid culture increases exosome secretion from mesenchymal stem cells. *Tissue Eng Regen Med.* 15, 427–436. DOI:10.1007/s13770-018-0139-5

Kim, J., Song, Y., Park, C.H., Choi, C., 2021. Platform technologies and human cell lines for the production of therapeutic exosomes. *Extracell Vesicles Circ Nucleic Acids.* 2, 3–17. DOI:10.20517/evcna.2020.01

Konishi, H., Hayashi, M., Taniguchi, K., Nakamura, M., Kuranaga, Y., Ito, Y., Kondo, Y., Sasaki, H., Terai, Y., Akao, Y., Ohmichi, M., 2020. The therapeutic potential of exosomal miR-22 for cervical cancer radiotherapy. *Cancer Biol Ther.* 21, 1128–1135. DOI:10.1080/15384047.2020.1838031

Kumar, D., Gupta, D., Shankar, S., Srivastava, R.K., 2015. Biomolecular characterization of exosomes released from cancer stem cells: possible implications for biomarker and treatment of cancer. *Oncotarget*. 6, 3280–3291. DOI:10.18632/oncotarget.2462

Logozzi, M., Di Raimo, R., Mizzoni, D., Fais, S., 2020. Immunocapture-based ELISA to Characterize and Quantify Exosomes in Both Cell Culture Supernatants and Body Fluids, first ed. Elsevier Inc, Rome.

Malhão, F., Macedo, A.C., Ramos, A.A., Rocha, E., 2022. Morphometrical, morphological, and immunocytochemical characterization of a tool for cytotoxicity research: 3D cultures of breast cell lines grown in ultra-low attachment plates. *Toxics.* 10, 415. DOI:10.3390/toxics10080415

Malm, M., Saghaleyni, R., Lundqvist, M., Giudici, M., Chotteau, V., Field, R., Varley, P.G., Hatton, D., Grassi, L., Svensson, T., Nielsen, J., Rockberg, J., 2020. Evolution from adherent to suspension: systems biology of HEK293 cell line development. *Sci Rep.* 10, 1–15. DOI:10.1038/s41598-020-76137-8

Morishita, M., Takahashi, Y., Nishikawa, M., Takakura, Y., 2017. Pharmacokinetics of exosomes-an important factor for elucidating the biological roles of exosomes and for the development of exosome-based therapeutics. *J Pharm Sci.* 106, 2265–2269. DOI:10.1016/j.xphs.2017.02.030

Panlilio, Y., 2020. *The Crucible: An Autobiography by Colonel Yay, Filipina American Guerrilla.* Rutgers University Press, New York. DOI:10.36019/9780813548203-024

Prasetyaningrum , P.W., Septisetyani, E.P., 2019. Teknik kultur sel mamalia tiga dimensi (3D) dalam bioteknologi kesehatan. *BioTrends.* 10, 9–17.

Putra, A.K., Askandar, B., Mustokoweni, S., 2015. Cleaved caspase-3 sebagai uji apoptosis pada kanker serviks IIB tipe sel skuamosa yang mendapat kemoterapi neoadjuvan cisplatin. *Maj Obstet Ginekol.* 23, 22. DOI:10.20473/mog.v23i1.2099

Rai, D.B., Medicherla, K., Pooja, D., Kulhari, H., 2023. Dendrimer-mediated delivery of anticancer drugs for colon cancer treatment. *Pharmaceutics.* 15, 801. DOI:10.3390/pharmaceutics15030801

Rao, X., Huang, X., Zhou, Z., Lin, X., 2013. An improvement of the 2ˆ(-delta delta CT) method for quantitative real-time polymerase chain reaction data analysis. *Biostat Bioinforma Biomath.* 3, 71–85.

Sakaue, T., Koga, H., Iwamoto, H., Nakamura, T., Ikezono, Y., Abe, M., Wada, F., Masuda, A., Tanaka, T., Fukahori, M., Ushijima, T., Mihara, Y., Naitou, Y., Okabe, Y., Kakuma, T., Ohta, K., Nakamura, K.I., Torimura, T., 2019. Glycosylation of ascites-derived exosomal CD133: a potential prognostic biomarker in patients with advanced pancreatic cancer. *Med Mol Morphol.* 52, 198–208. DOI:10.1007/s00795-019-00218-5

Sirenko, O., Mitlo, T., Hesley, J., Luke, S., Owens, W., Cromwell, E.F., 2015. High-content assays for characterizing the viability and morphology of 3D cancer spheroid cultures. *Assay Drug Dev Technol.* 13, 402–414. DOI:10.1089/adt.2015.655

Sutnick, A.I., Gunawan, S., 2020. Cancer in Indonesia. *J Am Med Assoc.* 247, 3087–3088. DOI:10.1001/jama.247.22.3087

Tian, H., Bharadwaj, S., Liu, Y., Ma, H., Ma, P.X., Atala, A., Zhang, Y., 2010. Myogenic differentiation of human bone marrow mesenchymal stem cells on a 3D nano fibrous scaffold for bladder tissue engineering. *Biomaterials.* 31, 870–877. DOI:10.1016/j.biomaterials.2009.10.001.

Tirino, V., Desiderio, V., Paino, F., De Rosa, A., Papaccio, F., Fazioli, F., Pirozzi, G., Papaccio, G., 2011. Human primary bone sarcomas contain *CD133* + cancer stem cells displaying high tumorigenicity *in vivo* . *FASEB J.* 25, 2022–2030. DOI:10.1096/fj.10-179036

Wan, L., Neumann, C.A., Leduc, P.R., 2020. Tumor-on-a-chip for integrating a 3D tumor microenvironment: chemical and mechanical factors. *Lab Chip.* 20, 873–888. DOI:10.1039/c9lc00550a

Zhang, X.L., Wang, W., Zhang, Y., Yu, W.T., Guo, X., Ma, X.J., 2006. Construction of *in vitro* multicellular tumor spheroid model with microencapsulation and its application in anticancer drug screening. *Chinese J Clin Rehabil.* 10, 72–75.

Zhang, J., Song Haijing, Dong, Y., Li, G., Li, J., Cai, Q., Yuan, S., Wang, Y., Song Haifeng, 2023. Surface engineering of HEK293 cell-derived extracellular vesicles for improved pharmacokinetic profile and targeted delivery of IL-12 for the treatment of hepatocellular carcinoma. *Int J Nanomedicine.* 18, 209–223. DOI:10.2147/IJN.S388916

Zhou, X., Natino, D., Zhai, X., Gao, Z., He, X., 2018. MicroRNA-22 inhibits the proliferation and migration, and increases the cisplatin sensitivity, of osteosarcoma cells. *Mol Med Rep.* 17, 7209–7217. DOI:10.3892/mmr.2018.8790