

# THE EFFECT OF CONDITIONED-MEDIUM HUMAN ADIPOSE-DERIVED MESENCHYMAL STEM CELL IN APOPTOSIS OF BLADDER CANCER CELLS

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## ABSTRACT

**Objective:** To determine the effect of conditioned medium human Adipose-Derived Mesenchymal Stem Cells (CM-hADMSC) on apoptosis of urothelial bladder cancer cells. **Material & Methods:** Bladder (5637) cancer cell lines cultured in conditioned media harvested from human adipose-derived mesenchymal stem cells (hADMSC). Flow cytometry tests were carried out using the Flowcytometry Acquisition cell sorting (FACS) Calibur to measure apoptosis. **Results:** There was a significant difference in the percentage of late apoptosis in the group receiving culture medium treatment: CM-hADMSC 1: 1 to the entire study group. Further analysis revealed no difference in the average percentage of late apoptosis in groups exposed to culture medium: CM-hADMSC 1: 2 and culture medium: CM-hADMSC 1: 4 ( $p > 0.05$ ). **Conclusion:** CM-hADMSC at a 1: 1 dose concentration to culture medium obtain a significant increase of apoptosis in bladder cancer cells.

**Keywords:** Bladder cancer, conditioned medium, adipose-derived mesenchymal stem cells, apoptosis.

## ABSTRAK

**Tujuan:** Mengetahui efek paparan conditioned medium human Adipose Derived Mesenchymal Stem Cells (CM-hADMSC) terhadap apoptosis sel kanker buli urothelial. **Bahan & Cara:** Sel kanker buli urothelial manusia tipe 5637 dibiakkan pada CM-hADMSC. Apoptosis diukur menggunakan Flowcytometry Acquisition cell sorting (FACS) Calibur. **Hasil:** Terdapat perbedaan signifikan persentase late apoptosis pada kelompok yang mendapatkan perlakuan medium kultur : CM-hADMSC 1:1 terhadap seluruh kelompok penelitian. Sedangkan pada dosis medium kultur : CM-hADMSC 1:2 tidak ditemukan adanya perbedaan persentase late apoptosis bila dibandingkan dengan kelompok perlakuan yang terpapar medium kultur : CM-hADMSC 1:4. **Simpulan:** Pemberian Conditioned medium hADMSC (CM-hADMSC) pada dosis 1:1 dengan medium kultur mengakibatkan terjadinya peningkatan apoptosis pada Sel Kanker buli urothelial.

**Kata Kunci:** Kanker buli, conditioned medium, adipose-derived mesenchymal stem cells, apoptosis.

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## INTRODUCTION

Bladder cancer is the most common urinary tract cancer with increased incidence. Bladder cancer is the 12<sup>th</sup> most common cancer with a 15% incidence rate per year in Indonesia.<sup>1-2</sup> Currently, bladder cancer management using intravesical

instillation, radical cystectomy, radiotherapy, and chemotherapy, depending on the type and stage of bladder cancer.

Mesenchymal stem cells (MSC) derived from adipose tissue, bone marrow, cord blood, and other tissues, have recently attracted much attention as potential therapeutic agents in various diseases

because of their trans-differentiation capacity.<sup>3</sup> MSC can migrate specifically to tumors and able to be used as a tumor-tropic vector with anti-cancer activity.<sup>4,5</sup> The use of the conditioned medium (CM) from MSC-sourced secretome stem-cell in regenerative medicine provides key advantages. CM is more economical than MSC since mass-production is possible and avoids excessive cell collection procedures.<sup>6</sup>

CM-hADMSC is known to have an anti-cancer effect. CM-hADMSC from the umbilical cord for glioma cells can significantly cause overexpression of any pro-apoptotic proteins such as Bad, Bax, Bim, Bid.<sup>7</sup> Another study has reported that hepatoma cells exposed by CM-hADMSC showing a decreased colony formation and proliferation.<sup>8</sup> The dualism effect is also found in CM-hADMSC which can inhibit the aggressive behavior of breast cancer cells in-vitro and reduce tumor growth in xenograft mice tumor models.<sup>9</sup>

Recently there was very little research on the effect of CM-hADMSC on bladder cancer cells, especially on urothelial cancer cells. In this study, we want to investigate the effect of CM-hADMSC, to measure viability and proliferation rates in urothelial bladder cancer cells exposed to human adipose mesenchymal stem cells.

## OBJECTIVE

To determine the effect of conditioned medium Adipose-Derived Mesenchymal Stem Cells (CM-ADMSC) exposure on apoptosis of urothelial bladder cancer cells.

## MATERIAL & METHODS

This research is an in vitro experiments using human bladder cell carcinoma type 5637 Cell lines Bladder Cancer 5637 are purchased from Cell Line Service (CLS) GmbH (Eppelheim, Germany). In this study, the study group was divided into 4 groups: untreated control group, Culture medium: hADMSC with 1:1 concentration group, Culture medium: hADMSC with 1:2 concentration group, and the Culture medium: hADMSC with 1:4 concentration group. Each group was replicated 6 times which is based on Federer's formula.

**Adipose tissue harvesting procedure.** An adipose tissue sample is taken from a pregnant woman who underwent an elective cesarean section

due to abnormal fetus location or narrow pelvis with no medical complication at Airlangga Hospital Surabaya. Adipose tissue harvesting procedure according to (Rachman et al., 2018) without modification.<sup>10</sup>

**Isolation and identification of MSC.** MSC are isolated from women who underwent a caesarean section and fat harvesting are performed intraoperatively. The adipose tissues then proceed as previously described by Rahman et al.

**Characterization and differentiation of hADMSC.** The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) proposes minimal criteria to define human MSC. First, MSC must be plastic-adherent when maintained in standard culture conditions. Second, MSC must express CD105, CD73, and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79? or CD19, and HLA-DR surface molecules. Third, MSC must differentiate to osteoblasts, adipocytes, and chondroblasts in vitro. Characterization and differentiation of the MSC procedure according to (Rachman et al., 2018) without modification.<sup>10</sup>

**Cells Culture.** 5637 cell line are low-grade primary bladder cancer. 5637 cells were cultured in RPMI. hADMSC were cultured in alpha-MEM supplemented with 10% FBS 10 ng/mL. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

**Conditioned Medium Harvesting.** hADMSC were cultured as previously described. When the cells grew to 80-90 % confluence at 3rd passage, the cell was cultured on free medium serum in 24 hours. Furthermore, 5 ml culture medium was aspirated, followed by the incubation period of 48h in CO<sub>2</sub> Incubator. Urothelial carcinoma cell culture was changed to CM-hADMSC mixed with culture medium followed by incubation period 48 hours.

**Apoptosis Analysis.** Apoptosis was measured using flow cytometry to quantify the levels of phosphatidylserine (PS) on the outer layer of the plasma membrane. The apoptotic test consisted of four groups: 1 control group (without treatment) and 3 treatment groups with CM-hADMSC for 24 hours, 48 hours, and 72 hours. The measurement begins with centrifugation of research cell samples with  $1-5 \times 10^5$  cells. Cells were washed using cold phosphate buffer saline (PBS) then the supernatant was discarded and pellets collected. Cell re-suspension was done using a binding buffer with a concentration of  $1 \times 10^6$

cells/mL. Reagents were prepared 100  $\mu$ L per sample. To each well 5  $\mu$ L staining solution was added then the sample was stirred gently. The mixed sample was incubated in a dark room at room temperature. Then 400  $\mu$ L 1 time-binding buffer in each tube, gently stir the tube again. Cells can be analyzed using flowcytometry no later than 1 hour after the treatment is done.

**Statistical Analysis.** Data obtained from Flow cytometry will be processed using the ANOVA parametric test. If the variant is homogeneous and the value of  $p < 0.05$ , the data will be analyzed by post hoc LSD test. If the variance is not homogeneous, the data will be analyzed by the Tamhane Post Hoc test. If the data processed does not meet the requirements, a non-parametric Kruskal-Wallis test will be conducted. If the value of  $p < 0.05$ , the data will be analyzed by the Mann-Whitney Post Hoc test. Statistical significance was considered when  $p < 0.05$ .

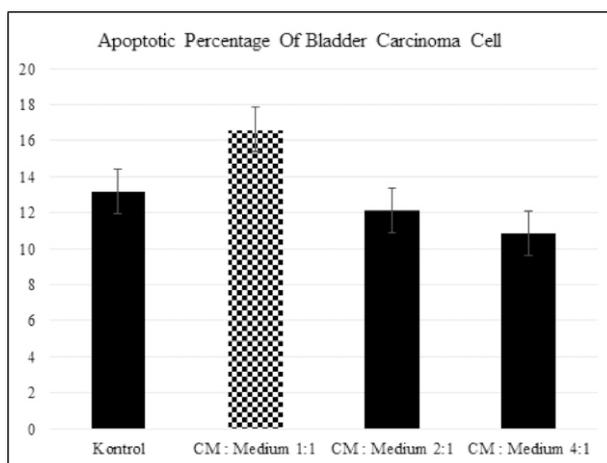
## RESULTS

From the percentage of late apoptosis data in all study groups, the data were normally distributed with  $p > 0.05$ . One-Way ANOVA results revealed that there were differences in the average percentage of late apoptosis in the study group ( $p < 0.05$ ). This indicated that there is a significant difference in the percentage of late apoptosis in at least one study group.

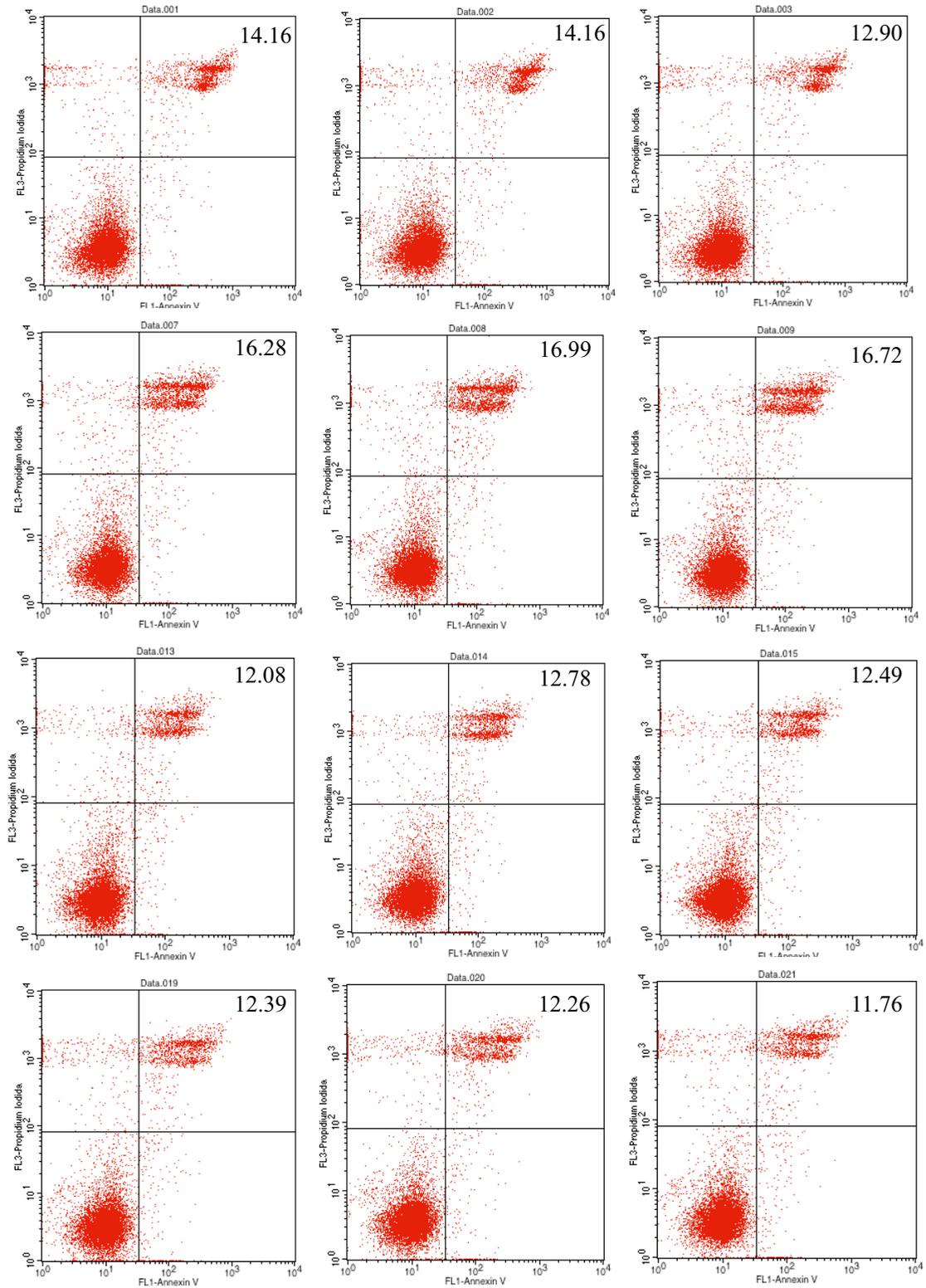
Post hoc analysis revealed that there was no difference in the average percentage of late apoptosis in groups exposed to culture medium: CM-hADMSC 1: 2 and culture medium: CM-hADMSC 1: 4 ( $p > 0.05$ ) (Figure 1). Further analysis revealed a significant difference in the percentage of late apoptosis in the group receiving culture medium treatment: CM-hADMSC 1: 1 to the entire study group. Whereas at the dose of culture medium: CM-hADMSC 1: 2 there was no difference in the percentage of late apoptosis when compared with the treatment group exposed to the culture medium: CM-hADMSC 1: 4.

**Table 1.** Post hoc test analysis between treatment groups of bladder carcinoma cells exposed to CM-hADMSC with different concentrations.

Group	Mean Difference	P-value
Control vs Cultured media : CM-hADMSC 1:1	3.38 (1.53-5.23)	0.03*
Control vs Cultured media : CM-hADMSC 1:2	1.06 (0.78-2.91)	0.29
Control vs Cultured media : CM-hADMSC 1:4	2.37 (0.38-4.79)	0.54
Cultured media : CM-hADMSC 1:1 vs Cultured media : CM-hADMSC 1:2	4.44 (3.75-5.13)	0.00*
Cultured media : CM-hADMSC 1:1 Vs Cultured media : CM-hADMSC 1:4	5.76 (3.57-7.94)	0.01*
Cultured media : CM-hADMSC 1:2 Vs Cultured media : CM-hADMSC 1:4	1.31 (0.86-3.49)	0.253



**Figure 1.** Percentage of late apoptosis Mean  $\pm$  SD in bladder carcinoma cells with conditioned medium exposure. There is a significant difference on group exposed to CM-hADMSC 1:1 compared with the control ( $p < 0.05$ ).



**Figure 2.** Fluowcitometry analysis of bladder carcinoma cell line. The number at the upper right quadrant shows the percentage of late apoptosis. The first row controls, the second row is CM-hAMSC 1:1 medium cell, the third row is CM-hAMSC 2:1 medium cell, the fourth row is CM-hAMSC 4:1 medium cell.

## DISCUSSION

Nowadays, many new biological reactions were found involving communication between cells through secreted substances, the secretome. Secretome is a collection of molecules secreted into extracellular space. Dissolved proteins, free nucleic acids, lipids, and extracellular vesicles are some examples of secretome cells produce. The use of MSC secretome as a therapy in regenerative medicine provides various advantages compared to the application of MSC directly. Secretome has the potential to increase the effectiveness of various cancer therapies today.<sup>9</sup> The use of secretomes is considered to be more economical and practical for clinical applications because it avoids invasive cell collecting procedures.<sup>11</sup>

The fact that the relation between stem cells and cancer cells was not fully understood. One study suggested that MSC inhibited the growth of human hepatocellular carcinoma cells (HepG2) and promoted apoptosis. This study also proved that the Wnt signaling pathway may have a role in tumor cell growth inhibition.<sup>12</sup> Research conducted by Otsu et al revealed that MSC has a cytotoxic and apoptotic effect in rat melanoma cells.<sup>13</sup> In our study, an effective dose that can induce significant apoptotic effects only on a 1: 1 comparison of culture medium: CM-hADMSC.

Cancer cells can replicate quickly and a constant mutation, these cells can adapt to an aggressive environment and survive after therapy. Cancer cells have "stemness" ability, it is the ability to continue their survival, to differentiate, to interact with the surrounding environment to maintain a balance, between quiescence, proliferation, and regeneration. Stemness is an important ability for the growth of cancer cells and is the main ability to survive. Besides cancer cells also have their stem cells or called cancer stem cells (CSC). CSC can be formed from a variety of stressor triggers such as chronic inflammation, accumulation of ROS (reactive oxygen species), and age. Cancer cells that have CSC can maintain progression and maintain survival which causes resistance to therapy.<sup>14</sup> Understanding the development and occurrence of cancer cell resistance can provide hope for more effective therapies. The MSC's dose may have a role to influenced tumor cell growth. Some studies that reported MSC promoting tumor growth, tend to use a higher MSC cell ratio than tumor cells.<sup>15</sup> Other researchers revealed when tumor cells and MSC co-injected in a higher MSC ratio, will promote tumor growth. This result shows that MSC can induce tumor growth in a dose-dependent manner.<sup>15-16</sup> Even though the result was statistically insignificant, this

study showed the dose-dependent effect of MSC may have a role in different apoptosis results. Further research can be done to determine the increase of the MSC dose of cancer cells.

Although other studies have shown good therapeutic results, the various types of CM being used were cultured under different conditions, from different passages, and different culture media.<sup>17</sup> The CM used in this study was carried out in 3<sup>rd</sup> passage MSC culture, with 80% confluency Abo-Aziza said that a confluence rate of 80% is a stage where cells can proliferate and differentiate properly.<sup>18</sup> A standardized method of production and validation in the use of CM from MSC is needed. However, the mechanism of interaction between stem cells and cancer cells was not fully understood. Further research are needed with more dose variants, more detailed methods that would be able to explain the exact mechanism and to obtain the lowest therapeutic dose of MSC that can be applied safely.

## LIMITATION

This study is in vitro study. Further research that focuses on the concentration of CM-hADMSC dose, duration of treatment, and mechanism of action is needed to improve research outcome.

## CONCLUSION

CM-hADMSC at a 1:1 dose concentration to culture medium obtain a significant increase of apoptosis in bladder cancer cells. Dose the addition of CM-hADMSC more than 1:1 ratio showed no increase of apoptosis compare to control group. Further research needs to be done on the effect of CM-HADMSC on bladder cancer cells with doses smaller than 1: 1 to find out the optimum dose of CM hADMSC resulting in increased apoptosis of urothelial bladder cancer.

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