RESEARCH ARTICLE

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The Effect of Avidin on Viability and Proliferation of Colorectal Cancer Cells HT-29

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Abstract

Objective: The aim of this study was to analyze the effect of avidin treatment on cell viability, proliferation and cyclin D1 expression in colorectal cancer cells HT-29. **Methods:** Colorectal cancer cell line HT-29 incubated with 50, 100, 150, and 200 µg/mL of avidin concentration during 24, 48, and 72 hours, then the cell viability and proliferation were analyzed. Each avidin concentration was conducted together with HT-29 cell line without avidin treatment as a control group. The cell viability was measured by MTS assay and the proliferation was measured by BrdU (5-bromo-2′-deoxyuridine) cell proliferation assay. According to cell viability and proliferation result, we determined the 100 µg/mL avidin concentration for analyzing mRNA and protein of cyclin D1. **Results:** We demonstrated that the viability and proliferation of HT-29 cells were significantly decreased in all concentration of avidin treatment compared to control. The cell proliferation showed larger reduction in avidin treatment rather than cell viability. This proves avidin could inhibit proliferation of colorectal cancer cell HT-29 quite well. The expression of cyclin D1, both mRNA and protein, was also significantly decreased after the avidin treatment group compared to control group, it supports the suppression of proliferation result. **Conclusion:** We concluded that avidin treatment could decrease cell viability and proliferation, accompanied by suppression of cyclin D1 expression in colorectal cells HT-29.

Keywords: Avidin- HT-29 Cells- viability- proliferation- Cyclin D1

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Introduction

Avidin, a protein found in egg whites, can bind biotin (vitamin B7/H) with high affinity (Kd = 10-15 M). Avidin-biotin interaction known to be the strongest non-covalent interaction in world (Holmberg et al., 2005). Because the strength and specificity of avidin-biotin interaction, so this pair widely used in several methods in molecular, immunological, and cellular assays (Bratthauer, 2010). Based on previous study, avidin can detract the availability of extracellular biotin, therefore impairing biotin-requiring enzymes and reducing cells viability and inhibiting its proliferation (Firakania et al., 2016; Zerega et al., 2001).

Several enzymes are required biotin as co-enzymes for their activities. Most of them are carboxylases such as acetyl-CoA carboxylase (ACC) that involved in fatty acid synthesis (Bhattacharjee et al., 2020; Mozolewska et al., 2020), pyruvate carboxylase (PC) involved in gluconeogenesis pathway (Kiesel et al., 2021; Ngamkham et al., 2020) and methylcrotonyl-CoA carboxylase (MCC) involved in branched-chain amino acids catabolism (Chen et al., 2021; He et al., 2020). All these enzymes play an important role in survivability and proliferation of cancer cells, so inhibition of this enzyme activity will suppress cancer cells progression. Beside those enzymes, bifunctional enzyme phosporibosylaminoimidazole carboxylase and also act as phosphoribosylaminoimidazole succino-carboxamide synthetase (PAICS) in de novo purine nucleotide synthesis, has carboxylase part which also requires biotin (Yin et al., 2018). De novo purine nucleotide synthesis also known as one of pathway actively used by cancer cells to produce purine in high amount to do cells division and PAICS itself reported to have important roles in cancer cells (Agarwal et al., 2020; Meng et al., 2018).

Cyclin D1 is an important protein in cell cycle regulation, it will form active complexes with cyclin dependent kinase (CDK) 4 and 6 that promote G1- to S-phase progression. Many studies prove that cyclin D1 act as key regulator in cell cycle progression, so it has been considered to be an oncogene in many cancers including colorectal cancer (Alao, 2007). Several studies reported that Cyclin D1 overexpressed in many cancers type and one-third or more in colorectal cancers. It also reported cyclin D1 expression is associated with poor prognostic

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factor in colorectal cancer patients (Y. Li et al., 2014). Cell line HT-29 is human colorectal adenocarcinoma cells with high expression of cyclin D1 which has important roles on cell proliferation (Mermelshtein et al., 2005).

Proliferation and metabolism are a critical way to inhibit tumor progression and metastasis. Suppressing of them will halt cancer cells growth and lowering its survivability. In the last decade, there were developed various less invasive treatments to control cancer cells progression. Previous study reported that avidin treatment reduced biotin availability and could halted PHA-induced human peripheral blood mononuclear cells (PBMC) proliferation and viability (Firakania et al., 2016). According to those study, we want to analyze the potential of avidin treatment in reducing of cancer cell viability and proliferation. One example of cancer cell type appropriately used in exploring of avidin effect is colorectal cancer cells, because avidin could be found in daily food source i.e. egg whites. So, this study revealed the avidin effect in colorectal cancer cells HT-29 viability and proliferation, also cyclin D1 expression in those cells.

Materials and Methods

This is an in vitro experimental study using human colorectal cancer cell line HT-29 treated by avidin. Cell line HT-29 was obtained from American Type Culture Collection (ATCC). It was conducted in Biochemistry and Biology Molecular Department Laboratory, Faculty of Medicine Universitas Indonesia, and Molecular Biology and Proteomics Core Facilities (MBPCF)-Indonesia Medical Education and Research Institute (IMERI), Faculty of Medicine Universitas Indonesia.

Colorectal cancer cells culture HT-29

Human colorectal cancer cells HT-29 were cultured in Dulbecco's Modified Eagle Medium (DMEM)-high glucose (PAN Biotech, Germany) containing 10% fetal bovine serum (FBS) (Biowest, France), 1% penicillin-streptomycin (Sigma-Aldrich, USA), and 1% amphotericin B (PAN Biotech, Germany). The cells were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO2. The cells were harvested after 80% confluence and could be used for further experiments.

Avidin Solution Preparation

Avidin (Sigma-Aldrich, USA) 1000 µg/mL stock solution was made by dissolving 10 mg avidin in 10 mL NaCl 0,9%. The solution then filtered aseptically by 0,22 µm millipore sterile filter. The stock solution can be diluted by culture medium to make the desirable concentration for experiments (50, 100, 150, and 200 µg/mL).

Cell Viability Assay

HT-29 cells cultured in 96-well plates with a density of 5 x 10^4 cells/well and incubated overnight. Afterwards, the cells were treated with 50, 100, 150, and 200 µg/mL of avidin concentration. All avidin treatments were conducted 3 replications and HT-29 cells without avidin treatments were used as control group. We measured the cell viability by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay using CellTiter 96® Aqueous Solution Cell Proliferation Assay Kit (Promega, USA). This assay was performed according to manufacturer's protocol. After the treatment and addition of reagents to each well, it was incubated at 37°C for 2 hours, then the absorbance was measured at 450 nm using ELISA reader. Cells viability was analyzed at 24, 48 and 72 hours of avidin incubation.

Cell Proliferation Assay

HT-29 cells cultured in 96-well plates with a density of 5 x 10^4 cells/well and incubated overnight. Afterwards, the cells were treated with 50, 100, 150, dan 200 µg/mL of avidin concentration. All avidin treatments were conducted 3 replications and HT-29 cells without avidin treatments were used as control group. BrdU Cell Proliferation ELISA kit (colorimetric) (Abcam, UK) was used for cells proliferation assay. This assay was performed according to manufacturer's protocol. The results were obtained after absorbance reading at 450 nm using ELISA reader. Cells proliferation was measured at 24, 48 and 72 hours of avidin incubation.

Relative mRNA expression of CCND1

HT-29 cells cultured in 12-well plates with a density of 5 x 10^5 cells/well and incubated overnight. Afterwards, the cells were treated with 100 µg/mL of avidin concentration then incubated in 24, 48 and 72 hours. Each avidin incubation was conducted 3 replications and HT-29 cells without avidin treatments were used as control group. The mRNAs were extracted from HT-29 cells by Quick-RNA™ Miniprep Plus Kit (Zymo Research, US). Quantitative RT-PCR was conducted by using SensiFAST™ SYBR® No-ROX One-Step Kit (Meridian Bioscience, USA) with human CCND1 (cyclin D1) Primer. Human 18sRNA was used as a housekeeping gene for calculating the relative expression of CCND1. Sequences of CCND1 primers are 5'-GAA GGA GAC CAT CCC CCT GA-3' as forward and 5'-GAA ATC GTG CGG GGT CAT TG-3' as reverse, it results 142 PCR product. Sequences of 18sRNA primers are 5'-AAA CGG CTA CCA CAT CCA AG-3' as forward and 5'-CCT CCA ATG GAT CCT CGT TA-3' as reverse, it results 155bp PCR product. Relative mRNA expression of CCND1 was calculated with the Livak method ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001).

Cyclin D1 Protein Level Assay

Cyclin D1 protein levels were measured by Human Cyclin D1 ELISA Kit (Abcam, UK). HT-29 cells cultured in 12-well plates with a density of 5 x 10^5 cells/well and incubated overnight. Afterwards, the cells were treated with 100 µg/mL of avidin concentration then incubated in 24, 48 and 72 hours. Each avidin incubation was conducted 3 replications and HT-29 cells without avidin treatments were used as control group. Cells were harvested and washed by phosphate buffer saline (PBS). After that, cells were extracted by Cell Extraction Buffer from Human Cyclin D1 Elisa Kit (Abcam, UK) and incubated on ice for 20 minutes. Cells then centrifuged and the supernatant transferred to a clean tube and it can be used for measuring of cyclin D1 protein and total protein concentration. The

supernatant then inserted to 96-wells plate from ELISA Kit to measure cyclin D1 protein levels. This assay was performed according to manufacturer's protocol. Total protein concentration was measured by read the absorbance at 280 nm (Christian-Warburg method) from the samples and bovine serum albumin (BSA) as the standard. Cyclin D1 levels were presented per mg protein total (ng/mg protein).

Statistical Analysis

This study obtained numerical data for cell viability, proliferation, relative mRNA expression of CCND1 and protein level of Cyclin D1. We analyzed the significant difference of cell viability and proliferation between control and various avidin concentration treatment group using One-Way ANOVA test. We also used independent t-test to analyze the significant difference of CCND1 mRNA expression and Cyclin D1 level between avidin treatment and control group. All statistical test was performed using SPSS software version 20.

Results

Viability of HT-29 cells after avidin treatment

Cell viability and proliferation were measured after avidin treatment to HT-29 cells. Cell viability number was calculated by divide live cells in avidin treatment group per live cells in control group, so we reported this data in percentage value. We found decreasing of HT-29 cell viability after avidin treatment in all concentration (50, 100, 150 and 200 ug/mL) and all incubation time (24, 48 and 72 hours). As we see in Figure 1, the number of cell viabilities in 24 and 48 hours of incubation were quite similar, around 70-80% for all avidin concentration. Cell viability in 72 hours incubation was around 80-90%, slight increase than 24 and 48 hours. Although the cell viability of HT-29 after avidin treatment was not greatly decreased, it was statistical significantly both in 24 hours ($p<0.001$), 48 ($p<0.05$) and 72 hours ($p<0.05$) incubation compare to control group.

Proliferation of HT-29 cells after avidin treatment

Cell proliferation number was also reported in percentage value that calculated by divide the absorbance of avidin treatment group by the absorbance of control group. We demonstrated that avidin treatment lead suppression of HT-29 cells proliferation in each concentration and all incubation time. The decreasing number of cell proliferation seem avidin dose dependent. In 24 hours of incubation, we found the smallest reduction of cell proliferation was 77.78% for 50 μg/mL avidin and the greatest was 25.40% for 200 μg/mL avidin. Similar pattern also shown in 48 and 72 hours of incubation, however the greatest reduction of cell proliferation was found in 48 hours. In 48 hours of incubation, the decreasing of cell proliferation was greatly significant ($p<0.001$) for all avidin treatment group and incubation time, although cell proliferation tends to increase in 72 hours of incubation (see Figure 2). We show the appearances of HT-29 cells culture growth both normal and avidin treatment group under inverted microscope 40x (see figure 3). We found that in general the number of cells is less in the avidin treatment than control group.

Relative mRNA expression of CCND1 and protein Cyclin D1 levels in HT-29 cells after avidin treatment

We also measured the expression of cyclin D1 in HT-29 cells after avidin treatment both in mRNA and protein level expression. We decided to use avidin concentration 100 ug/mL according to cell viability results. The previous results have shown that the cell viability treated by avidin with concentrations of 100, 150 and 200 showed a very significant decrease, but among three of them there was no significant differences (One-Way ANOVA test). So, we decided to use the lowest concentration of avidin (100 g/mL) to save reagents.

The relative mRNA expression of CCND1 showed significantly decreasing after 100 ug/mL avidin treatment in HT-29 cells both in 48 hours ($p<0.05$) and 72 hours ($p<0.001$) of incubation compared to control group. There is no difference of relative mRNA expression of CCND1

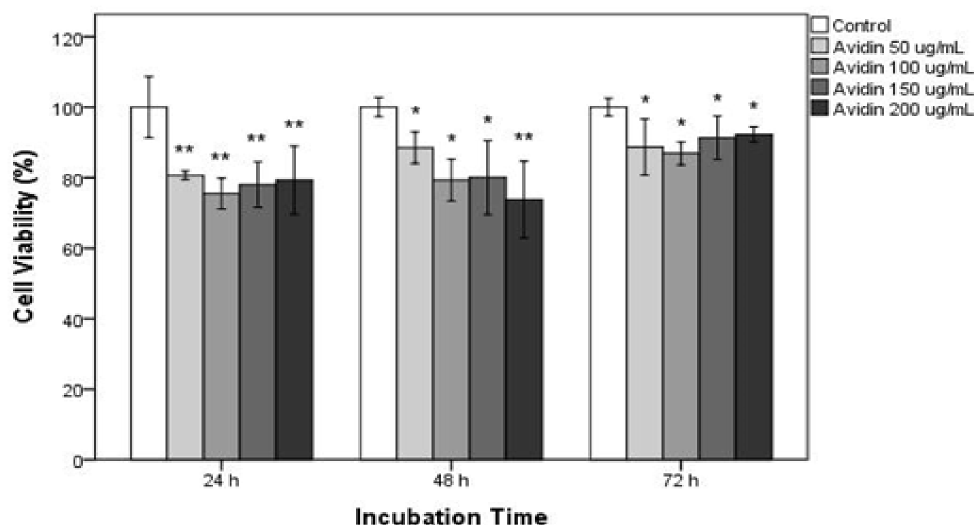

Figure 1. Cell Viability of HT-29 Cells Line after Avidin Treatment with Various Concentration (50, 100, 150 and 200 μg/mL) in 24, 48 and 72 hours of incubation. Significant difference compared to each control *$p<0.05$ and **$p<0.001$, One-Way Anova test.

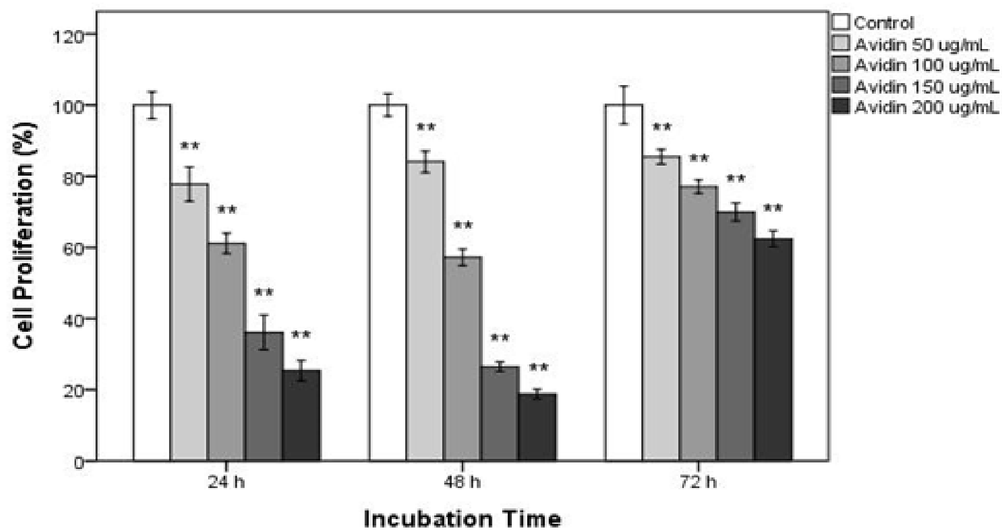

Figure 2. Cell Proliferation of HT-29 Cells Line after Avidin Treatment with Various Concentration (50, 100, 150 and 200 μg/mL) in 24, 48 and 72 hours of Incubation. Significant difference compared to each control **p<0.001, One-Way Anova test.

between avidin treatment in 24 hours of incubation and control (see Figure 4). This result support by the cyclin D1 protein levels finding, there are significant decreasing of cyclin D1 levels in HT-29 after 100 ug/mL avidin treatment both in 48 and 72 hours of incubation (p<0.001) compared to control group. While there is no difference of cyclin D1 levels between avidin treatment and control group in 24 hours of incubation (see Figure 5).

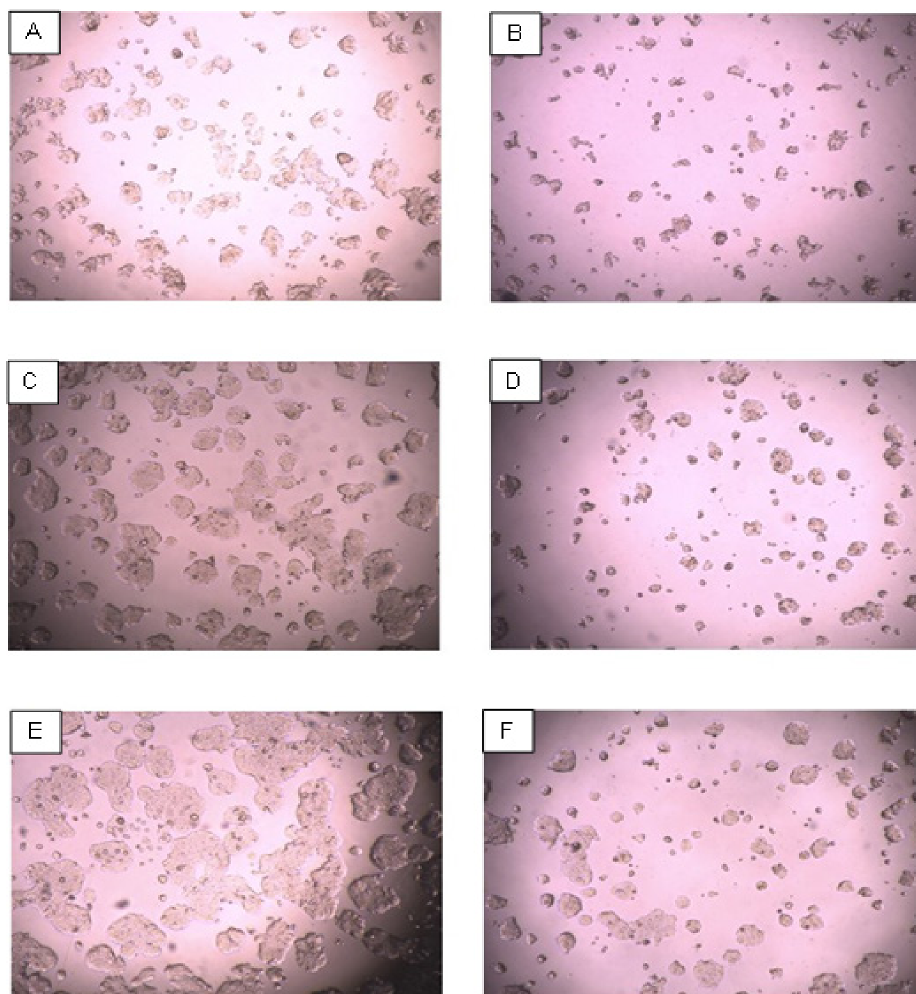

Figure 3. Cell Line Culture HT-29 under Inverted Microscope 40x; (A). control 24 hours, (B). 100 μg/mL avidin treatment in 24 hours incubation, (C). control 48 hours, (D). 100 μg/mL avidin treatment in 48 hours incubation, (E). control 72 hours, (F). 100 μg/mL avidin treatment in 72 hours incubation.

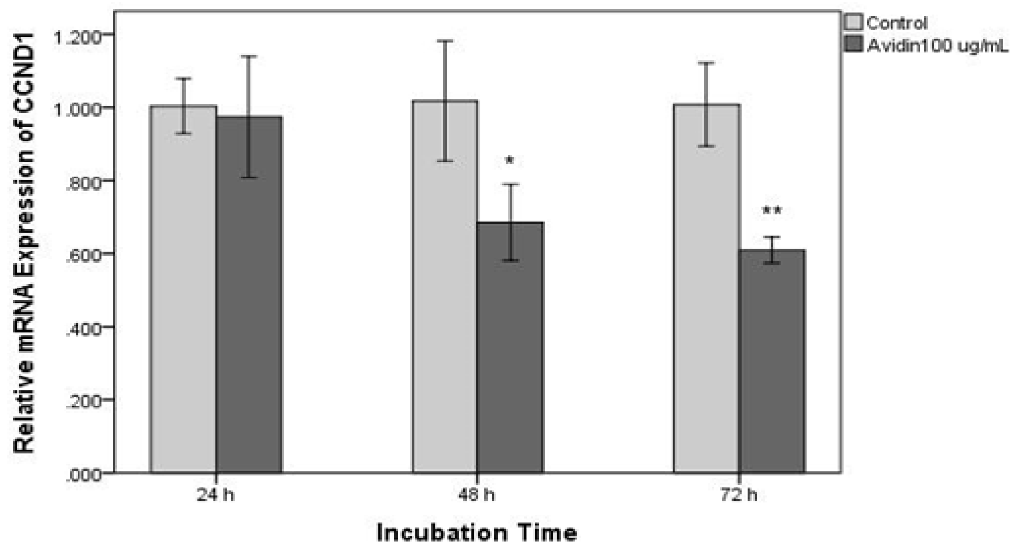

Figure 4. Relative mRNA Expression of CCND1 after 100 µg/mL Avidin Treatment on HT-29 Cells Line in 24, 48 and 72 hours of Incubation. Significant difference compared to control *p<0.05 and **p<0.001, independent t-test.

Discussion

Our findings in this study uncover new insight in searching of alternative cancer treatment. Significant decreasing of cell viability and proliferation after avidin treatment, making this compound is a promising agent in the development of cancer therapy. We demonstrated that cell viability and proliferation of HT-29 cells was significantly decrease after avidin treatment in all avidin concentration and all incubation time. If we see the alteration decreasing in cell proliferation is higher than cell viability. This showed that avidin treatment more suppress cell proliferation than cell viability. This is in accordance with the mechanism of avidin binds to biotin, causes vital enzymes that play a role in cell proliferation cannot work. We also found the maximum suppression of cell viability and proliferation occur in 48 hours incubation time. It might be caused the doubling time of HT-29 cells is 48 hours. So, if incubation longer than 48 hours the effectivity of avidin will decrease because the total HT-29 cells more abundant.

In this study we also demonstrated that cyclin D1 expression, both at mRNA and protein level, decreased in HT-29 cells after avidin treatment. Relative mRNA expression of CCND1 and cyclin D1 protein level were significantly lower compared to control, especially in 48 and 72 hours. This supports the result of declining proliferation of HT-29 cells after treated by avidin. Mechanism of avidin in decreasing cyclin D1 expression is still unclear, but this phenomenon might occur due to cell proliferation suppression. Other studies demonstrated that biotin required for biotinylation in histones (H2A, H3 and H4) by holocarboxylase synthetase and it influences some genes expression (Hassan and Zempleni, 2008; Narang et al., 2004). However, whether the biotinylation inhibition due to avidin treatment could affect the gene expression of cyclin D1, further study is required. Cyclin D1 plays a role in cell proliferation, especially regulate G1

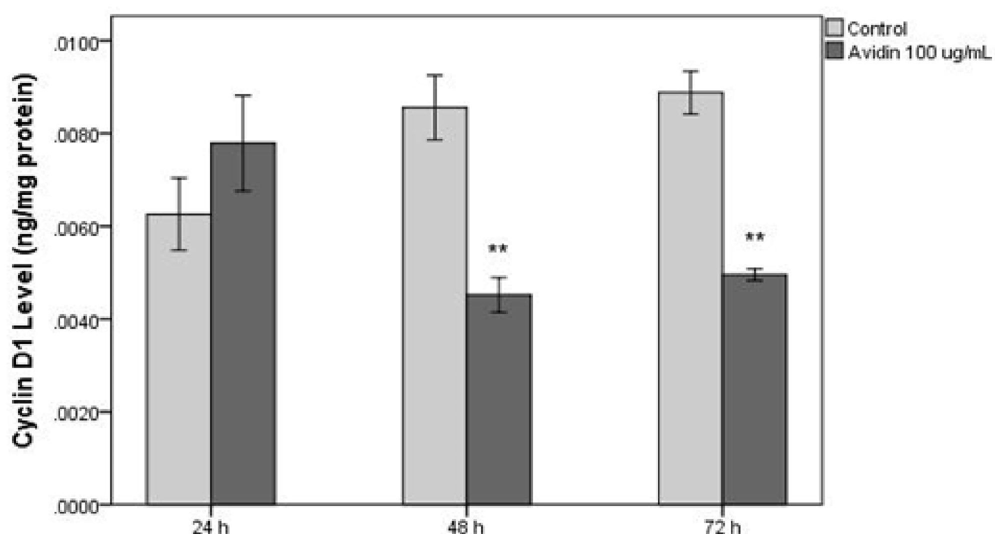

Figure 5. Cyclin D1 Protein Levels after 100 µg/mL Avidin Treatment on HT-29 Cells Line in 24, 48 and 72 hours of Incubation. Significant difference compared to control **p<0.001, independent t-test.

to S phase of cell cycle (Yang et al., 2006). Many studies showed that cyclin D1 correlated with colorectal cancer cells proliferation and malignant cells transformation (Albasri et al., 2019; Marcolino et al., 2020).

This study revealed the role of strong binding avidin to biotin will directly reduce cancer cells viability, proliferation, and cyclin D1 expression, because it reduces the availability of biotin for some important enzymes. Biotin takes an important role as coenzyme for a lot of notable enzymes in cell, particularly CO_2-using carboxylases like acetyl-CoA carboxylase (ACC) in fatty acid synthesis, pyruvate carboxylase (PC) in gluconeogenesis, methylcrotonyl-CoA carboxylase (MCC) in leucine metabolism, propionyl-CoA carboxylase in odd-chain fatty acid and amino acid metabolism, also carboxylase part of bifunctional enzyme, phosporibosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase (PAICS) in purine nucleotide synthesis (Tong, 2013; Waldrop et al., 2012).

ACC is a rate limiting enzyme in fatty acid (FA) synthesis. FA, on the other hand, is a building block for more complex lipids, as the vital source of structural membrane components, fuel source for growing and dividing cells, and second messengers in signal transduction (Bhattacharjee et al., 2020; Mozolewska et al., 2020). Cancer cells reportedly more rely on de novo FA synthesis pathway, while normal cells getting FA mostly from dietary sources (M. Chen and Huang, 2019). PC is an important enzyme for cellular energy metabolism by converting pyruvate to oxaloacetate in TCA cycle, key enzyme in gluconeogenesis, fatty acid synthesis, amino acid synthesis, and enhance protection from oxidative stress which are resulted from increasing metabolism. These functions enable metabolic plasticity to utilize any energy substrates depending on their availability, also support survival and growth cells, and metastases for some cases, the notable characteristic in cancer cells (Kiesel et al., 2021; Ngamkham et al., 2020).

MCC is a mithocondrial enzyme that reported to be an oncogene and connected with tumor formation and progression, especially methylcrotonyl-CoA carboxylase 2 (MCC2), a subunit of MCC. MCC2 overexpression reported in breast cancer and correlated with tumor formation and progression, also supporting leucine oncogenic function to promote hepatocellular carcinoma development and prostate cancer cells (Y. Y. Chen et al., 2021; He et al., 2020; Liu et al., 2019). Phosporibosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase (PAICS), is the less known enzyme that requires biotin as its coenzyme. PAICS is a key enzyme which catalyzes two essential steps in de novo purine nucleotide synthesis, which catalyzes 5-aminoimidazole ribonucleotide (AIR) to make carboxyaminoimidazole ribonucleotide (CAIR) in vertebrates (Li et al., 2007). Purine nucleotide synthesis known to be high in cancer cells than normal cells, caused by high requirement for DNA replication due to uncontrolled cell proliferation. PAICS enzyme has a carboxylase part and using CO_2 to catalyze the carboxylation, which is a similar trait to other biotin carboxylases (Tong, 2013). PAICS also reported to be involved in tumorigenesis, especially breast cancer and gastric cancer cells proliferation (Huang et al., 2020; Meng et al., 2018).

These biotin-requiring enzymes showed similar notable traits. First, they were high expressed and associated with some poor prognosis and tumor progression on cancer cells. They are also key enzymes and catalyze reactions which produce important components for energy sources, survival, oxidative stress protection, and cells division like fatty acid, oxaloacetate, and purine nucleotide (Tong, 2013). Reduced biotin availability as the result of avidin treatment to the medium cell caused the impairment of those enzymes that lead to reducing the cells viability and proliferation. A study reported that avidin bound extracellular biotin and affected acetyl-CoA carboxylase, thus regulated chick chondrocytes proliferation by interfering with fatty acid biosynthesis (Zerega et al., 2001). Another study also reported that avidin reduced biotin availability and could halted PHA-induced human PBMC proliferation and viability (Firakania et al., 2016).

These results revealed avidin as alternative agent, in reducing cells viability and inhibit cells proliferation, also decrease cyclin D1 expression in colorectal cancer cell lines HT-29. Moreover, avidin is easily to found in our daily food, it is contained in egg whites (Krkavcová et al., 2018). So, this can be another insight to use avidin as an anticancer candidate, especially colorectal cancer in the future. Although the exact mechanism has not known yet and need further research especially by in vivo experiment method. The study also can be expanded to avidin in egg whites as an ingestion therapy for cancer in digestive tract like colorectal cancer.

According to these results, we concluded that avidin could decrease colorectal cancer cell HT-29 viability and proliferation with a greater reduction in proliferation than viability. In addition, the expression of cyclin D1, both mRNA and protein, was also decreased in cells treated with avidin, this supports that there was an inhibition of cell proliferation after avidin treatment.

Author Contribution Statement

The authors confirm contribution to the paper as follows: study conception and design: Mohamad Sadikin; data collection: Muhammad Fakhri Ramadhan; analysis and interpretation of results: Syarifah Dewi and Murdani Abdullah; draft manuscript preparation: Syarifah Dewi and Muhammad Fakhri Ramadhan. All authors reviewed the results and approved the final version of the manuscript.

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Availability of data and materials

All data used and analyzed in this study are available from the corresponding author on reasonable request.

Conflict of interest

The authors declare no conflict of interest.

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