Anticancer Effects of *Escherichia Coli*-Derived Outer Membrane Vesicles against Colorectal Cancer Cells through Regulation of Apoptosis

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Abstract

Background: Colorectal cancer is one of the deadliest cancers with poor prognosis and limited treatment options. Microbiota and their metabolites, including toxins, have been shown to possess anti-tumor properties. Outer membrane vesicles (OMVs) from *Escherichia coli* may trigger inflammation in intestinal cells. We aimed to explore the anti-cancer effects of *E. coli*-derived OMVs on colorectal cancer cells by promoting apoptosis and pyroptosis, particularly through the over-expression of GSDMD in the CT-26 cell line.

Methods: In 2022, five non-replicating probiotic strains of *E. coli* were obtained from the Histogenotech Company in Tehran, and their OMVs were isolated and characterized. The CT-26 cell line was used to assess cell toxicity and the apoptotic effects of *E. coli*-derived OMVs. Real-time PCR and western blotting were performed to measure the expression of apoptosis and pyroptosis markers (GSDMD, NLRP3, and ROCK).

Results: The *E. coli* strains produced nanovesicles (5-200 nm in diameter). OMVs significantly reduced the viability of CT-26 cells and induced apoptosis at a concentration of 10 µg. The expression of GSDMD and NLRP3 was increased, while ROCK expression was downregulated in treated colorectal cells.

Conclusion: *E. coli*-derived OMVs can trigger inflammatory processes and induce negative effects on colorectal cancer cells. Further research is needed to evaluate their potential for colorectal cancer treatment.

Keywords: E. coli; CT-26; Colorectal cancer; Apoptosis; Inflammation

Introduction

The gastrointestinal system is linked to one of the most prevalent malignancies globally (1). The two most common types of these illnesses, gastric cancer (GC) and colorectal cancer (CRC), account for 1.8 and 1.0 million new cases per year, respectively, and account for 25% of cancer incidence globally (2). The third most prevalent malignancy globally, colorectal cancer (CRC) has a poor prognosis and a poor response to treatment (3, 4). Numerous factors, including as genetics, environment, lifestyle choices, dietary habits, and pharmaceutical regimens, might affect the initiation and progression of colorectal cancer (CRC) (5).

The anticancer properties of microbiota, their metabolites, and carcinogenic toxins have been

shown by earlier studies (6, 7). Consequently, *Escherichia coli* extracellular metabolites of short-chain fatty acids released revealed a strong regulatory effect on inflammatory responses and cytokine productions (8). It has been proposed recently that OMVs (outer membrane vesicles) generated from *E. coli* are a powerful factor in inducing inflammatory processes in intestinal epithelial cells (9). Living bacteria produce nano-sized vesicles known as OMVs at different phases of their development, especially during the logarithmic phase of growth. Phospholipids, poisons, nucleic acids, enzymes, and other proteins anchored in the cytoplasm, membrane, and outer membrane are among the items that these vesicles may carry (10, 11).

Extracellular vesicles (EVs) may suck into the cytoplasm of host cells and use clathrin-dependent endocytosis to transfer a multitude of virulence agents that can be taken up by different types of eukaryotic cells (12). One essential physiological process that takes place without the release of intracellular contents and without the following induction of an inflammatory response is apoptosis, or programmed cell death (13). This process was first identified by its morphological features, which included nuclear fragmentation, chromatin condensation, membrane blebbing, and cell shrinkage (14).

In the human body, there is a balance between cell division and proliferation under conditions of proper homeostasis. Thus, the dysregulation of apoptosis has major implications in carcinogenesis, and the imbalance between cell death and growth is thought to be the hallmark of malignant tumors (15). Furthermore, prior research suggested that deregulation of apoptosis in colorectal cells is a significant factor in cell division and the advancement of cancer (16).

Pyroptosis is an inflammatory mechanism of programmed cell death that inhibits the growth and migration of cancer cells while promoting inflammatory cell death in cancer (17). Nuclear GSDME over-expression in colorectal cancer limits cell proliferation and promotes apoptosis as a component of the immune response. Gasdermin E (GSDME) is a key indicator of py-

roptosis and closely interacts with caspase 3 during apoptosis (18).

We sought to ascertain the anti-cancer effects of *E. coli*-derived OMVs against colorectal cancer cells by over-expression of apoptosis and increase pyroptosis-related genes such GSDME in CT-26 cell line. This was done in light of the significance of colorectal cancer diagnosis and therapy.

Materials and Methods

Bacterial strains, OMV extraction, and OMV characterization

In 2022, five non-replicating probiotic strains of *E. coli* were obtained from the Histogenotech Company in Tehran, Iran and subsequently transferred to the laboratory at the Central Tehran Branch of Islamic Azad University, Tehran, Iran. The strains were cultured using MacConkey and Eosin Methylene blue agar, incubated for 48 h at 37 °C, and identified through biochemical assays (lactose fermentation, citrate, MR, VP, and indole tests). Each strain was stored at -70 °C in 20%-25% BHI medium. OMVs were isolated by culturing the bacteria in BHI medium for 72 h at 37 °C. After centrifugation, the bacteria were removed, and the remaining liquid was filtered. OMVs were further isolated using ultracentrifugation at 200,000 g for 3 h at 4 °C (12). The purified OMVs were rinsed with sterile distilled water and stored at -80 °C for future analysis. The size and morphology of the OMVs were confirmed using scanning electron microscopy (SEM) and dynamic light scattering (DLS).

Cell Culture and Viability Assay

E. coli-derived outer membrane vesicles (OMVs) were applied to CT-26 cells, a mouse colorectal carcinoma cell line, to assess their impact on colorectal cancer cells. The CT-26 cells, obtained from Histogenotech (Tehran, Iran), were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Cells were incubated at 37 °C with 5% CO_2 and 95% humidity. To

evaluate the cytotoxicity of OMVs, an MTT assay was performed. CT-26 cells were plated in 96-well plates and treated with various concentrations of OMVs (10 μg/mL) for 24 h. After adding MTT reagent and incubating for 4 h, absorbance was measured at 560nm using an ELx808 microplate reader.

CT-26 Cells Treatments and Apoptosis Assays

In order to expose CT-26 cells to OMVs, the cells were cultivated in 6-well plates at a concentration of 50,000 cells per well and kept in an incubator at a temperature of 37 °C with a CO2 concentration of 5% for a duration of 24 h. The CT-26 cells were subjected to a concentration of 10 μg/mL of outer membrane vesicles (OMVs) derived from *E. coli* for a period of 24 h. Furthermore, the control group consisted of untreated CT-26 cells. The experiment was conducted on three separate occasions. The cells were used for molecular analyses and apoptotic assessment following a duration of 24 h. The FITC Annexin V Apoptosis Detection Kit from Sigma, USA, was used according to the specified directions to assess the apoptotic effects of *E. coli*-derived outer membrane vesicles (OMVs).

Quantitative Real-Time PCR

The mRNA levels of NLRP3, ROCK, and GSDMD genes were measured using quantitative real-time PCR with a StepOne Plus PCR instrument and bioFACT™ 2X Real-Time PCR master mix. Total RNA was extracted from treated and untreated cells using a commercial kit, and cDNA was synthesized using a cDNA synthesis kit. GAPDH was used as an internal control. The expression levels of the genes were analyzed with specific primers, and melting curve analysis was performed after each run. Gene expression was quantified using the 2-ΔΔCt method, with results presented as fold changes relative to untreated cells. The experiments were conducted in triplicate (Table 1).

Table 1: Primers were used for real-time PCR assay in this study

Gene	Sequences	Product size (bp)	Reference
GSDMD	F: AGAGGCGATCTCATTCCGGT R: GACAACATCAC-TCTGCCCTGA	236	This study
Rock1	F: TCCTCGAACGCTGTCAACAA R: AACCCTGAAAA-GCCTGTGGT	203	This study
NLRP3	F: TTTGCTGGAAGAG-GAGGCAG R: GGCCAAAGAGGAATCG-GACA	152	This study
m-GAPDH	F: GGTCCCAGCTTAGGTTCATCA R: ACTGTGCCGTTGAATTT-GCC	233	This study

Western Blotting for Protein Expression Levels

Western blotting was used to assess the protein expression of GSDMD, a key marker for pyroptosis and apoptosis, in CT-26 cells treated with E. coli-derived OMVs. Protein was extracted using RIPA buffer, and concentrations were measured with the Bradford assay. Samples were treated with 4× protein sample dye, heated, and separated by SDS-PAGE. Proteins were transferred to a PVDF membrane, blocked with 4% non-fat milk

in PBS-T. The membranes were incubated overnight at 4°C with primary antibodies targeting GSDMD and GAPDH (as a loading control). After washing, secondary antibodies conjugated with HRP were applied, and protein bands were detected using the Odyssey Infrared Imaging System.

Statistical Analysis

The statistical analysis was performed using GraphPad Prism software version 8 (GraphPad Software, Inc., La Jolla, CA, USA). The one-way analysis of variance (ANOVA) and t-test were used to ascertain the disparities among the different groups. Unless explicitly stated differently, the findings are shown as the mean ± standard error of the mean (SEM) of a minimum of three studies. Statistical significance was established for differences when the p-value was less than 0.05. Additionally, $*P<0.05$, $**P<0.01$, $***P<0.001$, and $****P<0.0001$ were used to denote increasing levels of significance.

Results

E. coli strains released spherical OMVs

We obtained five different strains of *E. coli* from the Tehran, Iran-based company Histogenotech (Tehran, Iran). Biochemical and microscopic examination revealed gram-negative rods with a positive MR test result, an indole reaction, and a negative VP and citrate reaction. Figure 1 depicts the typical size range of 5-200 nm for more than 90% of isolated OMVs, as established by the use of scanning electron microscopy and dynamic light scattering. Scanning electron microscopy data was used to verify the polymorphic morphological structure of OMVs.
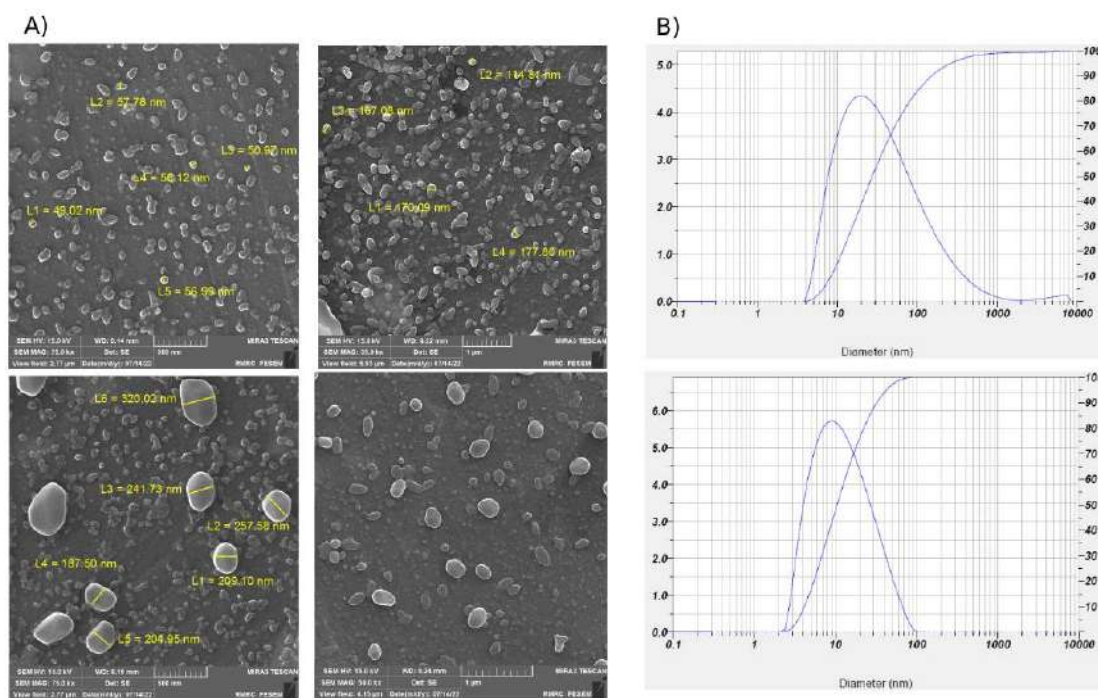

Fig. 1: Characterization of *E. coli*-derived OMVs. (A) Scanning electron microscopic (SEM) images indicated the spherical and oval shaped vesicles in different sizes. (B) Physicochemical characteristics of H. pylori-derived OMVs based on DLS

Effects of E. coli-derived OMVs on CT-26 cell viability

The cytotoxic effects of OMVs produced by *E. coli* on the CT-26 cell line were determined using direct microscopic examination and a cell viability assay. OMVs derived from *E. coli* strains 1 and 3 significantly decreased the number of viable CT-26 cells when exposed to a dose of 10 μg/ml over the whole co-infection period, in compari-

son to the results seen in untreated control cells after 48 h. Therefore, the extracellular vesicles (OMVs) derived from strains 2, 4, and 5, when present at a concentration of 10 μg/mL, did not have a significant impact on the survival ability of the CT-26 cell line (Fig. 2). Consequently, OMVs derived from each strain examined were used for further investigations.
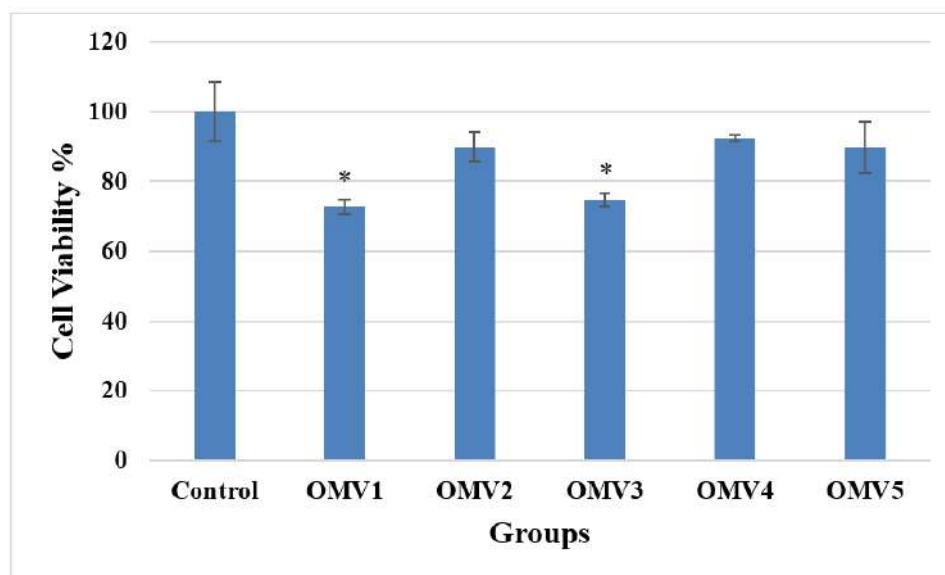

Fig. 2: Effects of different concentrations of the OMVs extracted from *E. coli* strains on cell viability of the CT-26 cells

E. coli-derived OMVs induced apoptosis in CT-26 cell line

A flow cytometry test was conducted to differentiate between apoptotic cells and healthy cells. This test used annexin V, a protein that binds to phospholipids and interacts with the externalized phosphatidylserine (PS) on the plasma membrane

after apoptosis. The CT-26 cell line, when exposed to OMVs from strains 1, 2, 3, 4, and 5, exhibited rates of early and late apoptosis of 29.25%, 16.6%, 4.185%, 5.307%, and 11.08% respectively after 48 h (Fig. 3), compared to the untreated control cells.
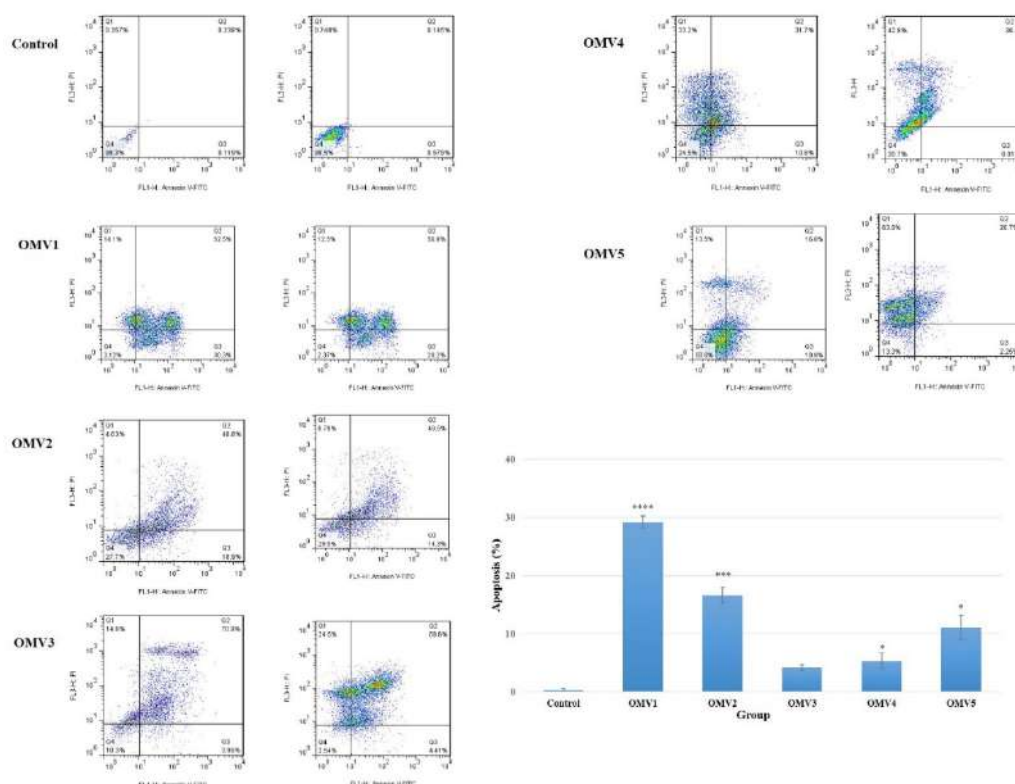
Fig. 3: Effects of OMVs extracted from different *E. coli* strains (10 μg/mL) on the CT-26 cells apoptosis induction. As shown OMVs from strains number 1, 2, 4, and 5 significantly inducted apoptosis in CT-26 cells. Data presented as means ± standard error (SEM) for three independent experiments. Data are shown as the mean ± SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$ and by post-hoc one-way ANOVA statistical analysis

OMVs were significantly increased expression level of GSDMD and NLRP3 and highly decreased ROCK mRNA expression

To study the expression of inflammatory markers in treated CT-26 cells, the mRNA expression levels of GSDMD, NLRP3, and ROCK were determined by qRT-PCR. The GAPDH gene was used as an internal control. Figure 4 shows that treatment with OMVs dramatically enhanced GSDMD and NLRP3 gene expression while downregulating ROCK expression. OMVs have the capacity to modify CT-26's inflammatory response markers.
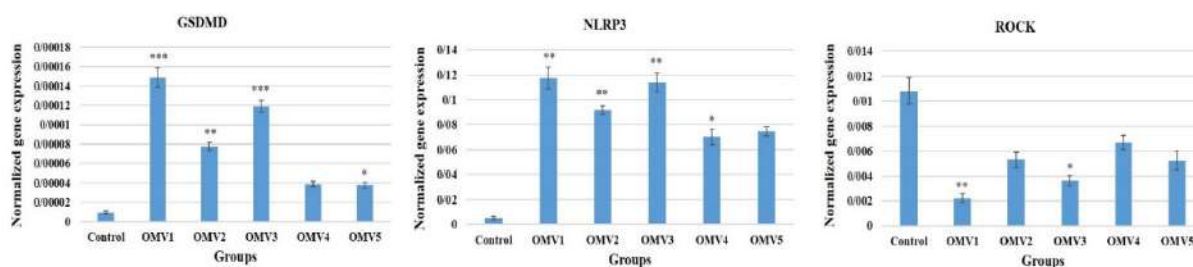

Fig. 4: Gene expression of inflammatory-related markers in treated CT-26 cells, upon treatment with 10 μg/ml of *E. coli* OMVs. Relative gene expression of fibrosis markers (GSDMD and NLRP3) was markedly increased after treatment of CT-26 cells with 10 μg/ml of *E. coli* OMVs. mRNA level of ROCK was decreased in CT-26 cells treated with 10 μg/ml of *E. coli* OMVs. Data presented as means ± standard error (SEM) for three independent experiments. Data are shown as the mean ± SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$ and by post-hoc one-way ANOVA statistical analysis

Protein expression dramatically mirrored the mRNA expression

Western blot analysis was performed to evaluate the effects of *E. coli*-derived stellate on response markers. Following exposure of CT-26 cells to OMVs, the GSDMD marker was analyzed. *E. coli*-derived OMVs upregulated the GSDMD reg-

ulator in CT-26 cells compared to untreated hepatic stellate cells. When compared to the CT-26 cells that were not treated, the immunoblotting findings were in accordance with the real-time PCR result, indicating that the expression of the inflammatory response protein was activated (Fig. 5).
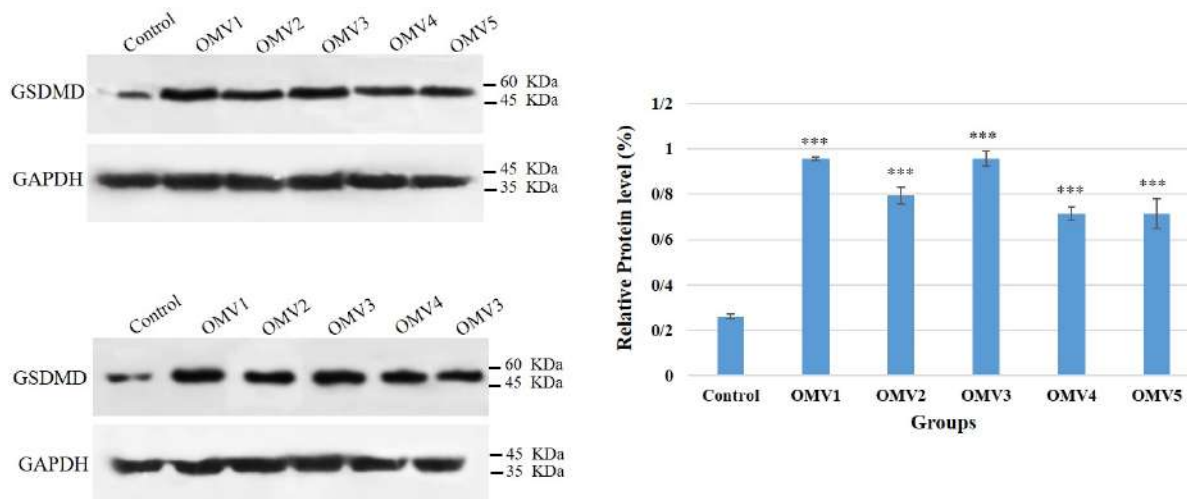
Fig. 5: Immunoblotting result of GSDMD protein expressions. As it shown GSDMD protein expression of CT-26 cell increased after treatment with *E. coli* OMVs. Data presented as means ± standard error (SEM) for three independent experiments. Data are shown as the mean ± SEM. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ by post hoc one-way ANOVA statistical analysis. Scale bar: 20 μm, magnification 200X

Discussion

One of the most prevalent cancers worldwide, colorectal cancer is associated with the makeup of the gut microbiota (19). Previous studies have demonstrated the relevance of the interactions between intestinal epithelial cells and the gut microbiota in a number of gastrointestinal illnesses (20, 21). Furthermore, in patients with a variety of gastrointestinal cancers, microbial dysbiosis is a crucial stage in the proliferation, carcinogenesis, and tumorigenesis of intestinal cells (22).

Prior studies have shown that systemically given *E. coli* probiotic strains have the powerful capacity to colonize and multiply in malignant tissues in animal models, indicating that tumor-targeted therapy is an important method in the treatment of cancer (23, 24). Furthermore, due to the cru-

cial role that patients' nutrition plays in their recovery from colorectal cancer, the use of probiotics and prebiotics in treatment regimens has expanded dramatically (25). Moreover, bacterial outer membrane vesicles have attracted considerable interest in recent years as a basis for innovative cancer therapy approaches, owing to their hazardous contents (10, 11, 26).

In this work, we investigated the impact of OMVs produced from *E. coli* probiotic strains on CT-26 cells' tumor growth using many methods, including flow cytometry, qPCR, Western blotting, and MTT test. This work included the isolation of outer membrane vesicles (OMVs) from many distinct strains of probiotic *E. coli* bacteria. Our research has shown that OMVs have a spherical shape and have a diameter ranging from 5 to 200 nm. Park et al. conducted a study on the lethal consequences of *E. coli*-derived outer

membrane vesicles (OMVs) in animal models, which is consistent with our own findings. The study verified the existence of a spherical structure with two layers in the OMVs that were obtained from the liquid portion of cultured *E. coli*. An examination employing dynamic light scattering revealed that the bulk (75.0%) of the isolated vesicles had diameters ranging from 25 to 50 nm (27). OMVs produced from *E. coli* MG1655 protected bacteria against colistin and melittin, both of which are cell membrane-targeting antibiotics. Furthermore, *E. coli* purified OMVs ranged in size from 50 to 80 nanometers. Furthermore, the adoption of various techniques to separate OMVs had little effect on their size or shape (28). Furthermore, ultracentrifugation was employed to separate outer membrane vesicles (OMVs) from the *E. coli* K-12 strain.

Transmission electron microscopy (TEM) examinations indicated that the OMVs had a diameter of around 100 nm. The researchers discovered OmpA in isolated OMVs and demonstrated its potential to increase the protein expression of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) markers in macrophages (29). The study demonstrated the harmful effects of E. coli-derived outer membrane vesicles (OMVs) on the CT-26 colorectal cancer cell line at a concentration of 10 μg/mL, showing their cytotoxic potential for therapeutic use. This finding aligns with previous research by Jin et al., which explored the effects of OMVs on neuroblastoma. Both in vitro and in vivo studies confirmed the anticancer properties of OMVs without significant side effects. OMVs were found to reduce cell stemness, induce DNA damage, trigger apoptosis, and cause cell cycle arrest, thereby inhibiting tumor growth. The study suggests that bacterial OMVs could be promising candidates for future cancer therapies (30). Furthermore, Kim et al. explore the possibility of bacterial outer membrane vesicles as therapeutic agents for cancer treatment via immunotherapy. Their study uncovers the impressive capacity of bacterial outer membrane vesicles to effectively initiate long-lasting immune responses against cancer, perhaps leading to complete eradication of existing tumors without any adverse effects. The vesicles are distributed in a methodical way and possess a unique capability to specifically target and accumulate in the tumor tissue. Consequently, they promote the synthesis of antitumor cytokines CXCL10 and interferon-γ (31).

Our study demonstrated that bacterial outer membrane vesicles (OMVs) have the potential to serve as effective immunotherapeutic agents for treating various cancers without noticeable side effects. We showed that *E. coli*-derived OMVs can trigger apoptosis in CT-26 colorectal cancer cells, as confirmed by annexin V-based flow cytometry. Treatment with different types of *E. coli*-derived OMVs at 10 μg/mL induced apoptosis in these cells. Additionally, a study by Alizadeh et al. examined the anticancer effects of *E. coli* probiotic strains on the HT-29 human colorectal cancer cell line. Their research found that these probiotic strains promote programmed cell death by modulating the Bax/Bcl and AKT/PTEN signaling pathways, decreasing Bcl-xL and AKT1 levels, and increasing PTEN and Bax levels (32). These findings together demonstrated that OMVs derived from probiotic strains effectively inhibit the growth of cancer cells.

Furthermore, extracellular vesicles generated from *E. coli*, at a concentration of 10 μg/mL, significantly increased the expression of the GSDMD and NLRP3 genes. Bacterial outer membrane vesicles (OMVs) possess the capacity to stimulate inflammatory pathways inside cancer cells. According to our study, Yang et al. (33) shown that *E. coli* OMVs effectively recruit neutrophils to the lung by stimulating the production of IL-8/CXCL1 from endothelial cells via the TLR4- and NF-$\varkappa$B-dependent pathway. The in vivo investigation demonstrated that intraperitoneal administration of *E. coli* OMVs resulted in an augmentation of CXCL1 production. CXCL1 is a chemotactic factor for neutrophils that exhibits functional similarity to human IL-8. In addition, the researchers observed a significant increase in the number of neutrophils entering the lung tissues of mice after the delivery of outer membrane vesicles (OMVs) obtained from *E. coli*.

Conclusion

In recent years, there has been a lot of interest in employing probiotics and their products, including OMVs (released structures), to cure cancer. However, a lack of documentation in this subject makes it difficult to fully understand their benefits and drawbacks. OMVs derived from *E. coli* probiotic strains had the capacity to stimulate inflammatory responses in CT-26 cell lines, decrease the viability of cancer cells, and trigger apoptosis in cancer cells. In order to have a clear understanding of the effect of OMVs generated by *E. coli* on the elimination of cancer cells, more research and in vivo studies are required.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflicts of Interest

The authors declare that they have no competing interests.

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