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Escherichia Coli Outer Membrane Vesicles Induced DNA Double-Strand Breaks in Intestinal Epithelial Caco-2 Cells

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

A **Zhou Ling**
A **Chen Dayong**
A **Yu Denggao**
A **Wang Yiting**
A **Fang Liaoqiong**
A **Wang Zhibiao**

State Key Laboratory of Ultrasound Engineering in Medicine Co-Founded by Chongqing and the Ministry of Science and Technology, College of Biomedical Engineering, Chongqing Medical University, Chongqing, P.R. China

Corresponding Authors:

Wang Zhibiao, e-mail: wangzb@cqmu.edu.cn, Fang Liaoqiong, e-mail: lqfang06@163.com

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Background:

Recent studies have shown that *Escherichia coli* induced digestive tract diseases may be related to outer membrane vesicles (OMVs) induced intestinal double-strand breaks (DSBs) in intestinal epithelial cells. This study aimed to compare the impact of OMVs forces on DSBs in intestinal epithelial Caco-2 cells, and provide a new treatment for digestive diseases caused by *E. coli*.

Material/Methods:

E. coli OMVs were prepared and co-cultured with Caco-2 cells. The uptake of OMVs by Caco-2 cells was observed by confocal microscopy. The γ -H2AX protein was detected by western-blot. The DSBs caused by OMVs was detected by single cell gel electrophoresis.

Results:

The particle size analyzer showed that the average diameters of OMVs centrifuged at 20 000×g and 50 000×g were 217.5±7.29 nm and 186.3±6.59 nm ($P<0.05$), respectively. Transmission electron microscopy of the OMVs revealed a lipid bilayer structure with a variety of different sizes. Confocal fluorescence microscopy revealed that OMVs almost completely entered Caco-2 cells after 24 hours. The ratio of γ -H2AX protein band gray value normalized data in the OMVs centrifuged at 20 000×g and 50 000×g, and the control group (without OMVs) were 2.23±0.18, 1.58±0.20, 1±0.30 ($P<0.05$), respectively, while DNA levels of the comet tail (TailDNA%, TDNA%) were 72.21±14.61%, 23.11±4.98%, and 1.02±1.41% ($P<0.05$), respectively. The corresponding DNA damage was categorized as high (grade 3), moderate (grade 2), and no damage (grade 0).

Conclusions:


Different sizes of OMVs induced different degrees of DNA damage in intestinal epithelial Caco-2 cells.

MeSH Keywords:

DNA Damage • *E. coli* OMV • Intestinal Epithelial Caco-2 Cells

Full-text PDF:

<https://www.basic.medscimonit.com/abstract/index/idArt/913756>

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Background

Outer membrane vesicles (OMVs) are characterized by a phospholipid bilayer membrane structure and are released from the outer membrane of bacterial cell walls [1,2]. Almost all bacterial cells secrete OMVs. Bacteria release OMVs by disrupting a connection between the outer membrane and peptidoglycan, inducing local membrane curvature, and changing specific protein levels [3–5]. OMVs from different sources have different functions, including regulating host immune response [6], performing vaccine function [7–9], transporting biomolecules [10,11], protecting bacterial cells [12,13], assisting biofilm formation [14,15], and responding to physical and chemical stresses [16].

Escherichia coli is closely associated with a number of digestive diseases and is harmful to human health if intestinal flora becomes disordered or unbalanced. *E. coli* can adhere to intestinal epithelial cells and then release substances that have negative impact on digestive tract, including diarrhea, gastrointestinal discomfort, intestinal bleeding, and intestinal adhesions [17–22], which in turn may cause inflammatory bowel disease, irritable bowel syndrome, or even intestinal cancer.

Recent studies have shown that one possible cause of *E. coli*-induced digestive tract diseases may be related to OMV-induced intestinal double-strand breaks (DSBs) in intestinal epithelial cells. *E. coli* secrete OMVs, which are induced by intestinal epithelial receptor cells to cause DNA DSBs in intestinal epithelial cells [23]. Tyrer et al. found that *E. coli* OMVs enter intestinal epithelial Caco-2 or HT-29 cells and release virulence factors, such as virulence proteins, heat labile endotoxins (LTs), and enterotoxins which can cause DNA DSBs in intestinal epithelial cells [24] and trigger a corresponding digestive tract disease. DSB induces H2AX phosphorylation in the conserved region of serine 139 at the C-terminus to form γ -H2AX [25–28]. Therefore, detection of γ -H2AX has become the gold standard for DSB detection. A variety of physical, chemical, and biological factors that can induce the formation of γ -H2AX have been identified to date. Ivashkevich et al. [29] used a γ -H2AX kit to detect DNA damage, while Janaki et al. [30] used single cell gel electrophoresis (comet assay) to detect DNA DSBs.

In this study, *E. coli* OMVs were prepared using intestinal epithelial Caco-2 cells and 2 centrifugal forces. The sizes of the 2 OMVs were compared to determine whether they caused the same damage to the Caco-2 cells. Using this OMV data, the pathogenicity of *E. coli* can be explained, which provides a new treatment for digestive diseases caused by *E. coli*.

Material and Methods

Cell suspension

Intestinal epithelial Caco-2 cells were purchased from the cell bank of the Chinese Academy of Sciences, and *E. coli* (ATCC 25922) was acquired from Qingdao Rishui Biotechnology Co., Ltd. Caco-2 cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, penicillin (100 μ g/mL), and streptomycin (100 μ g/mL) and were subsequently inoculated in 25-cm² culture dishes and incubated at 37°C and 5% CO₂. The cells were allowed to grow to an approximate confluence of 80% before passaging.

Extraction of OMVs by ultracentrifugation

The LB broth medium was autoclaved for 20 minutes (120°C, 100 Kpa) and then cooled to room temperature. A single colony on the *E. coli* culture plate was inoculated and cultured at 37°C and 180 rpm for 10 days. The supernatant was collected by centrifugation at 1500 \times g for 15 minutes at 4°C using benchtop centrifuge 5810R (Eppendorf, USA). The supernatant was then filtered through a 0.45- μ m filter (Merck Millipore), followed by a 0.22- μ m filter (Merck Millipore), to remove residual bacteria. The OMVs were obtained by centrifugation at 20 000 \times g and 50 000 \times g for 1.5 hours at 4°C in an ultra-speed refrigerated centrifuge (HITACHI 55P-72, Hitachi, Japan), washed, resuspended in 1 mL of HEPES buffer, and finally stored at 4°C for future use.

Particle size analysis

Samples of OMVs (15 μ g) obtained using the 2 different centrifugal forces were each dissolved in 1 mL of HEPES buffer and vortexed for 1 minute to allow OMVs to distribute evenly. The size distribution of *E. coli* OMVs was then analyzed using the Malvern particle size analyzer (Zeta SIZER 3000HS, Malvern, UK).

Transmission electron microscopy

The OMVs obtained by centrifugation at 20 000 \times g and 50 000 \times g for 1.5 hours were gently mixed with 1 mL of 4% glutaraldehyde, fixed for 2 hours (4°C), then washed 3 times. The OMVs were then fixed with 1% osmium tetroxide for 2 hours. OMVs were dehydrated using conventional ethanol and acetone gradient, followed by impregnation, embedding, and polymerization with epoxy resin to prepare semi-thin sections (0.5 μ m) for subsequent imaging using a light microscope. Ultra-thin sample sections (60 nm) were then prepared and stained using uranium acetate and lead citrate for electron microscopy observation.

Observation of OMV uptake by Caco-2 cells using confocal microscopy

Dio dye (6 μL , 10 mg/mL) was mixed with 20 μg of the OMV suspension and stained in a 37°C incubator for 30 minutes. This was followed by addition of phosphate-buffered saline (PBS) and washing at 50 000 \times g for 90 minutes. Dio-traced OMVs and Caco-2 cells were co-cultured for 0 hours, 12 hours, and 24 hours and then fixed with 4% paraformaldehyde. The cells were then stained with 6 μL of DAPI dye (10 mg/mL) for 10 minutes at room temperature. One drop of anti-fluorescent culturing agent and appropriate amount of PBS were then added for observation of OMV uptake by the Caco-2 cells using confocal fluorescence microscope TCS-SP2 (Leica, Germany).

Western blot detection of γ -H2AX protein expression

Caco-2 cell were plated into 6-well plates, incubated with DMEM containing 10% FBS, penicillin (100 $\mu\text{g}/\text{mL}$), and streptomycin (100 $\mu\text{g}/\text{mL}$) for 12 hours, and treated with 20 μg OMVs from the 20 000 \times g group, the 50 000 \times g group, and the control group for 24 hours, respectively. The total protein from each group was extracted and the loading buffer was boiled for 90 seconds to denature the protein. A 20- μL protein sample was obtained for SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). The protein was then fixed under a constant electric field of 250 mA followed by transfer to a nitrocellulose membrane. Skim milk powder (5%) was used for blocking overnight at 4°C, to which the corresponding primary antibody was added to incubate the proteins at room temperature overnight. The membrane was washed 3 times with Tris-buffered saline with Tween 20 (TBST) buffer for 10 minutes each time. Horseradish peroxidase-labeled secondary antibody was then added and incubated for 1 hour at room temperature and washed three times with TBST buffer for 10 minutes each time. The experiment was repeated 3 times and results were analyzed after color development by ECL.

Single cell gel electrophoresis

A total of 1×10^5 Caco-2 cells were inoculated in 25-cm² culture flasks and 20- μg OMV samples were added to the 20 000 \times g OMV group and the 50 000 \times g OMV group after adhering for 4 hours. OMVs were not added to the control group. The 3 groups were cultured for 24 hours. The cells were digested and washed once with PBS and the concentration of the cells was adjusted to 10^3 – 10^4 cells/mL after centrifugation. Three layers of rubber sheets were prepared by sequentially using 150 μL of 1% normal-melting agarose, 10 μL of PBS containing 1000 cells, 100 μL of 0.8% low-melting agarose, and 100 μL of 0.5% low-melting agarose. The coverslips were removed, and the slides were immersed in fresh cell lysate and incubated for at least 1 hour at 4°C. The slides were then rinsed twice with PBS, placed in a horizontal

electrophoresis tank, and immersed in alkaline running buffer for 20 minutes. Electrophoresis was carried out for 20 minutes at 25 V and 300 mA. The slides were then neutralized with Tris-HCl (pH 7.5) for 15 minutes. Then, 50 μL of the 30- $\mu\text{g}/\text{mL}$ ethidium bromide solution was added dropwise to each slide, which was subsequently covered with a cover glass, and stained for 20 minutes in the dark. The electropherogram was observed and photographed using a fluorescence microscope and the experiment was repeated 3 times. The Comet Assay Software Pect (CASP 1.2.3 beta 1) image analysis software was used to analyze each comet image, where $X \pm SE$ was used to represent the full length (CL), tail length (TL), tail moment of the comet (TM), and olive tail (OTM) for further calculating the tailing factor. According to the tail DNA concentration (TailDNA%) in tail cells, the degree of DNA damage was divided into 5 levels: grade 0, no damage (normal cells) and cell damage rate <5%; grade 1, low DNA damage with cell damage between 5% and 20%; grade 2, moderate damage with cell damage between 20% and 40%; grade 3, high damage with cell damage between 40% and 90%; and grade 4, severe damage with cell damage >95%.

Statistical analysis

The experimental data were processed by SPSS10.0 and *t*-test analysis was performed between independent samples. The results were expressed as mean \pm standard deviation. $P < 0.05$ indicated statistical significance.

Results

Impact of centrifugal force on OMV size

E. coli OMVs collected using 2 centrifugal forces were detected by Malvern particle size analyzer. The average particle sizes for the 20 000 \times g group, the 50 000 \times g OMV groups were 217.5 ± 7.29 nm and 186.3 ± 6.59 nm, respectively (Figure 1A). The 2 groups were significantly different from each other ($P < 0.05$), as shown in the corresponding histograms (Figure 1B, Table 1). Transmission electron microscopy clearly showed *E. coli* OMVs' lipid bilayer with spherical structures of different sizes and diameters <1 μm (Figure 1C).

Confocal fluorescence observation of OMV in Caco-2 cells

Confocal fluorescence microscopy showed that the control group without OMVs had only blue nuclei. When Dio-OMVs were added to the Caco-2 cells, OMVs were initially scattered around the cells and located far from the nuclei. After 12 hours of co-culture, green fluorescence of Dio-OMVs was observed around nuclei, indicating that some Dio-OMVs entered the Caco-2 cells. After 24 hours of co-culture, the uptake of Dio-OMVs by Caco-2 cells was significant (Figure 2).

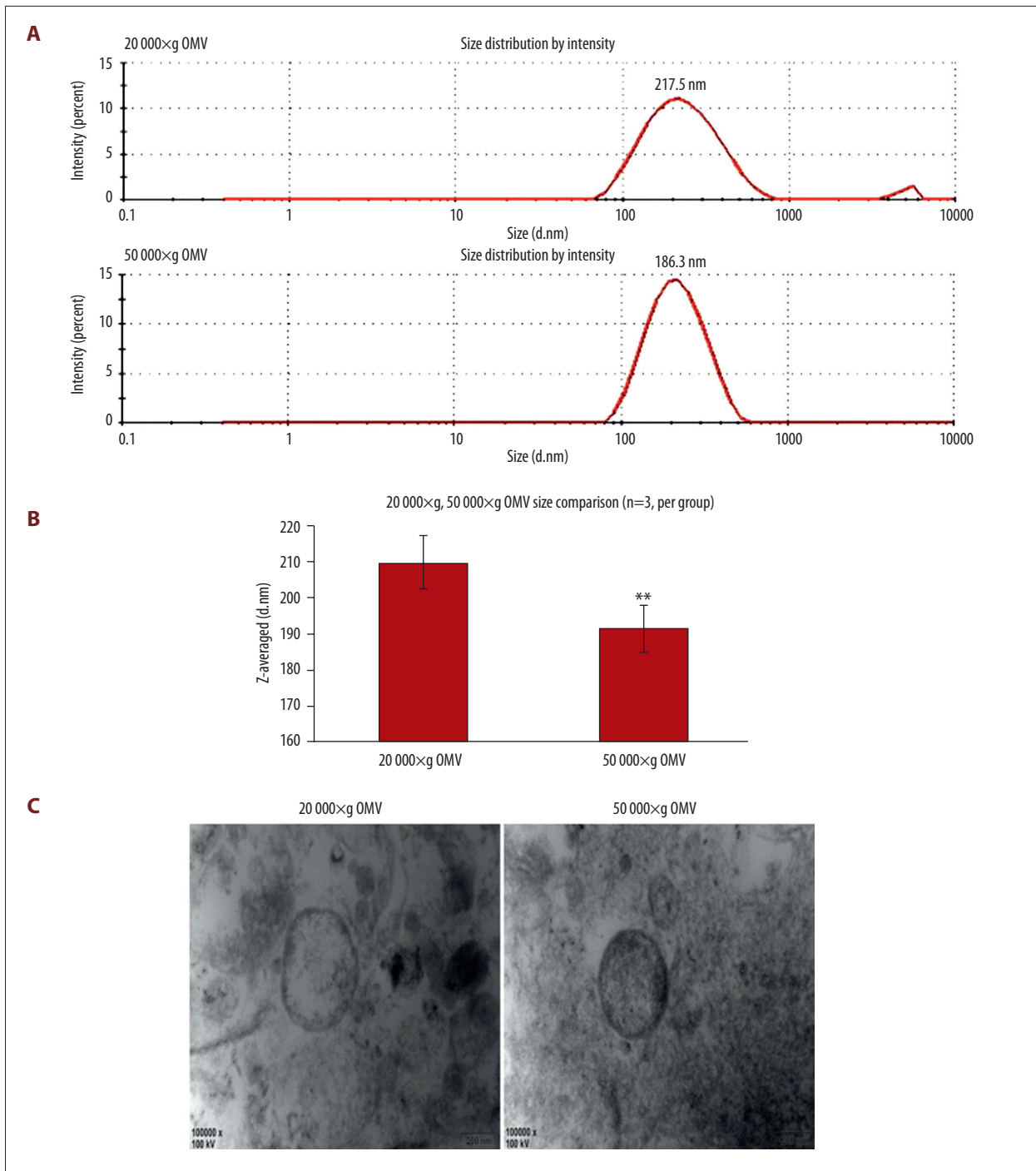


Figure 1. *Escherichia coli* outer membrane vesicles (OMV) size and morphology. **(A)** OMV obtained by 20 000×g and 50 000×g centrifugation (n=3). **(B)** Size comparison between 20 000×g and 50 000×g OMV groups (n=3, per group). **(C)** 20 000×g and 50 000×g OMV group morphology.

Table 1. OMV size analysis.

Centrifugal force/g	Mean/nm	SD
20 000×g	217.5	7.29
50 000×g	186.3	6.59

SD – standard deviation.

Impact of different OMV sizes on the expression of γ -H2AX protein in Caco-2 cells

E. coli OMVs collected using 2 different centrifugal forces were co-cultured with Caco-2 cells for 24 hours. Western blot was used to analyze the ratio of γ -H2AX protein band gray value normalized data in Caco-2 cells and the results showed that expression of γ -H2AX protein in the 20 000×g group, the 50 000×g group, and the control groups were 2.23 ± 0.18 , 1.58 ± 0.20 , and 1 ± 0.30 ($P<0.05$), respectively (Figure 3A). The expression of γ -H2AX protein statistical analysis of the relative expression levels of γ -H2AX protein revealed significant differences among the 3 groups ($P<0.05$) (Figure 3B).

Impact of OMV size on DNA damage in Caco-2 cells

Caco-2 cells were co-cultured with 20 000×g OMVs and 50 000×g OMVs for 24 hours. Fluorescence imaging was performed using single-cell gel electrophoresis. The results showed that the tail of 20 000×g OMV group was longer than that of and 50 000×g OMV group, while the control group exhibited almost no tail (Figure 4A). The Comet Assay Software Pect (CASP 1.2.3 beta 1) (Table 2) was used to perform statistical analysis of TailDNA% for the 3 groups (Figure 4B). The results showed that TailDNA% of the 20 000×g OMVs group, the 50 000×g OMVs group, and the control groups were $72.21\pm 14.61\%$, $23.11\pm 4.98\%$, and $1.02\pm 1.41\%$, respectively. Significant differences were observed among the 3 groups ($P<0.05$). The degree of DNA damage for the 3 groups were high (grade 3), moderate (grade 2), and no damage (grade 0), respectively.

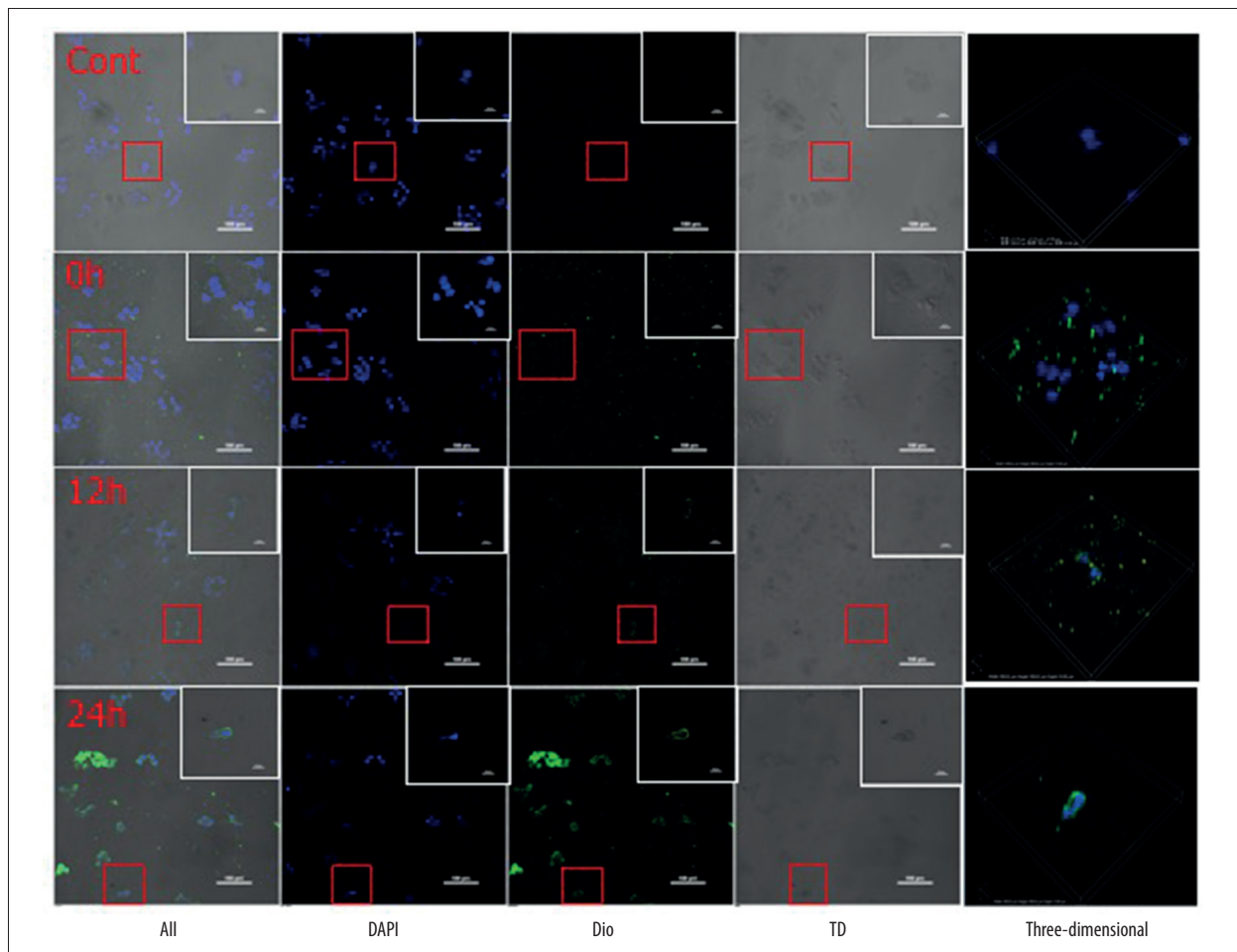


Figure 2. Confocal microscopy imaging of Dio-labeled outer membrane vesicles (OMVs) taken up by Caco-2 cells.

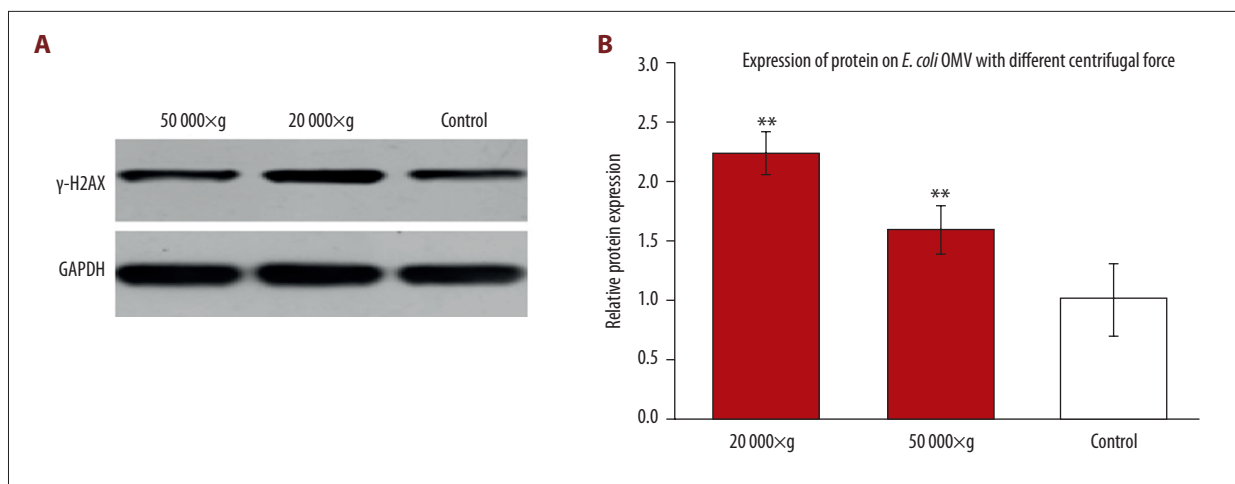


Figure 3. γ -H2AX protein levels. (A) Expression of γ -H2AX protein. (B) Expression of γ -H2AX protein in *Escherichia coli* outer membrane vesicles (OMVs) obtained with different centrifugal forces.

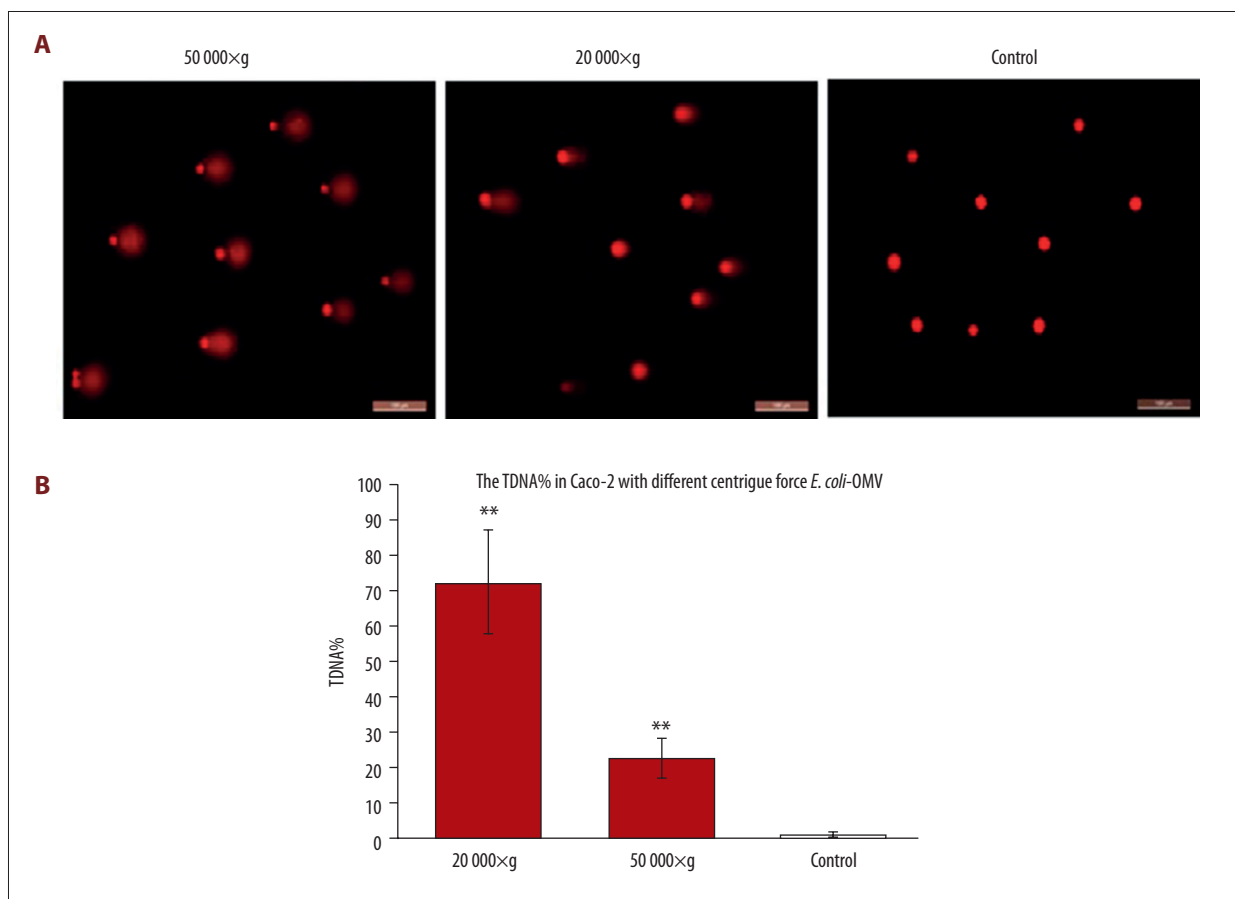


Figure 4. Impact of outer membrane vesicle (OMV) size on DNA damage in Caco-2 cells. (A) Single-cell gel electrophoresis; (B) TailDNA% in Caco-2 cells for *Escherichia coli* OMVs obtained with different centrifugal forces.

Discussion

This study revealed that OMVs prepared by different centrifugal forces are significantly different from each other. Transmission

electron microscopy imaging showed that although the morphology of OMVs was similar between the 20 000×g OMVs group and the 50 000×g OMVs group, the particle size distribution of the 2 OMV groups was different, as detected by particle

Table 2. Software analysis of tail length (TL), comet full length (CL), tail moment (TM), and olive tail length (OTM) to calculate tailing DNA rate.

Name	Group	Tail DNA%	Tail length	Comet length	Tail moment	Olive tail moment
20 000×g	1	67.1583	110	197	0.00663	43.9357
	2	73.1448	118	169	0.0796036	52.8612
	3	86.3233	124	171	0.00576123	61.7247
50 000×g	1	19.4544	53	126	10.3108	8.58909
	2	28.7827	65	140	18.7	14.0588
	3	21.0904	56	139	11.8106	10.5368
Control	1	0.999067	99.8	0.22104	73.8742	3
	2	9.13098	97.3465	2.65345	86.3	3
	3	0.903067	99.808	0.192041	107.041	3

size analyzer. Furthermore, OMVs of different sizes showed distinct capability for DNA damage in Caco-2 cells, which was likely due to distinct OMV inclusions. Currently, differential centrifugation combined with filtration, ultrafiltration, and ExoQuick kits, among others, is the main method for preparation of vesicles of different sizes [31–34]. Each procedure has its advantages and disadvantages and there are no methods that can generate vesicles of a precise size. Instead, vesicles that fall within a certain size range can be created. Vesicles with larger particle sizes can be obtained by low-speed centrifugal force. The vesicle particle size decreases with increasing centrifugal force. High speed centrifugal force impacts the extraction of small and high-purity vesicles. In summary, OMVs prepared by using distinct centrifugal forces combined with other methods have different properties, thus carrying distinct information and performing distinct biological functions.

Single cell gel electrophoresis (comet assay) is commonly used to detect DNA DSBs. In this study, cell lysis was performed based on the principle of degeneration, which further unwinds DNA. Nuclei are then electrophoresed, and damaged DNA fragments migrate out influenced by the electric field. The undamaged DNA remains in the nuclei and migrates slowly. Eventually, staining with fluorescent DNA dye allows to observe the DNA shape and migration patterns and to analyze DNA damage via comet tails and the percentage of tail DNA. The degree of DNA DSB induced by OMVs can then be determined. Duthie et al. [35] used Caco-2 cells as an *in vitro* model of human colon cells and employed alkaline single-cell gel electrophoresis to determine DNA damage. Follmann et al. [36] used alkaline gel electrophoresis to determine DNA DSBs in bovine colon cells. In our study, alkaline double-cell gel electrophoresis was used to analyze the DNA DSBs of Caco-2 cells. The results showed that *E. coli* OMVs induced significant DNA DSBs (DNA tailing) in Caco-2 cells, which was consistent with results reported by Robichová and Anderson et al. [37,38]. Further studies have shown that

both types of OMVs can induce DNA DSBs in Caco-2 cells with different degree of DNA damage.

DNA DSB is an important type of DNA damage. It is first signaled by the phosphorylation of serine residue of histone H2AX at the C-terminus near breakpoint, forming γ -H2AX [39–41]. Phosphorylated γ -H2AX can transfer DNA damage signals fast, leading to activation of phosphorylation of downstream molecules and triggering a series of biological cascades and cytological responses. The results of this study showed that *E. coli* OMVs induced higher levels of γ -H2AX protein in Caco-2 cells. Different levels of OMVs induced Caco-2 cells to express distinct levels of γ -H2AX protein. *E. coli* OMVs also induced changes in γ -H2AX protein levels expressed in Caco-2 cells, which may be the result of DNA damage in Caco-2 cells.

In conclusion, *E. coli* OMV-induced DNA damage in intestinal epithelial Caco-2 cells may be one of the causes for *E. coli*-induced digestive diseases. This study revealed that OMVs centrifuges at 20 000×g were larger than OMVs centrifuged at 50 000×g, and that 20 000×g OMVs more severely damaged Caco-2 cell DNA, which may be related to OMV inclusions.

Conclusions

Our study revealed that *E. coli* could secrete OMVs, and that different sizes of OMVs induced different degrees of DNA damage in intestinal epithelial Caco-2 cells.

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References:

- Livshits MA, Khomyakova E, Evtushenko EG et al: Corrigendum: Isolation of exosomes by differential centrifugation: theoretical analysis of a commonly used protocol. *Sci Rep*, 2016; 6: 21447
- McBroom AJ, Johnson AS, Kuehn MJ: Outer membrane vesicle production by *Escherichia coli* is independent of membrane instability. *J Bacteriol*, 2006; 188(15): 5385–92
- Kuehn MJ, Kesty NC: Bacterial outer membrane vesicles and the host-pathogen interaction. *Genes Dev*, 2005; 19(22): 2645–55
- Alves NJ, Turner KB, Medintz IL et al: Emerging therapeutic delivery capabilities and challenges utilizing enzyme/protein packaged bacterial vesicles. *Ther Deliv*, 2015; 6(7): 873–87
- Kulp A, Kuehn MJ: Biological functions and biogenesis of secreted bacterial outer membrane vesicles. *Annu Rev Microbiol*, 2010; 64(2): e783–89
- Perez Vidakovic MLA, Jendholm J, Mörgelin M et al: B cell activation by outer membrane vesicles – a novel virulence mechanism. *PLoS Pathogens*, 2010; 6(1): 543–47
- Pol LD, Stork M, Ley PVD: Outer membrane vesicles as platform vaccine technology. *Biotechnol J*, 2015; 10(11): 1689–706
- Kim OY, Hong BS, Park KS et al: Immunization with *Escherichia coli* outer membrane vesicles protects bacteria-induced lethality via Th1 and Th17 cell responses. *J Immunol*, 2011; 190(8): 4092–102
- Jagannadham MV, Chattopadhyay MK: Role of outer membrane vesicles of bacteria. *Resonance*, 2015; 20(8): 711–25
- Bomberger JM, Maceachran DP, Coutermarsh BA et al: Long-distance delivery of bacterial virulence factors by *Pseudomonas aeruginosa* outer membrane vesicles. *PLoS Pathogens*, 2009; 5(4): e1000382
- Cañas MA, Giménez R, Fábrega MJ et al: Outer membrane vesicles from the probiotic *Escherichia coli* Nissle 1917 and the commensal ECOR12 enterointestinal epithelial cells via clathrin-dependent endocytosis and elicit differential effects on DNA damage. *PLoS One*, 2016; 11(8): e0160374
- Vella BD, Schertzer JW: Understanding and exploiting bacterial outer membrane vesicles. *Pseudomonas*, 2015; 217–50
- Kulkarni HM, Jagannadham MV: Biogenesis and multifaceted roles of outer membrane vesicles from Gram-negative bacteria. *Microbiology*, 2014; 160(10): 2109–21
- Kouokam JC, Sun NW: Outer membrane vesicle-mediated export of a pore-forming cytotoxin from *Escherichia coli*. *Toxin Reviews*, 2008; 25(1): 31–46
- Lee EY, Bang JY, Park GW et al: Global proteomic profiling of native outer membrane vesicles derived from *Escherichia coli*. *Proteomics*, 2007; 7(17): 3143–53
- Baumgarten T, Sperling S, Seifert J et al: Membrane vesicle formation as a multiple-stress response mechanism enhances *Pseudomonas putida* DOT-11e cell surface hydrophobicity and biofilm formation. *Appl Environ Microbiol*, 2012; 78(17): 6217–24
- Levine MM: *Escherichia coli* that cause diarrhea: Enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. *J Infect Dis*, 1987; 155(3): 377–89
- Besser RE, Lett SM, Weber JT et al: An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157: H7 in fresh-pressed apple cider. *JAMA*, 1993; 269(17): 2217–20
- Roubos-van den Hil PJ, Nout MJ, Beumer RR et al: Fermented soya bean (tempe) extracts reduce adhesion of enterotoxigenic *Escherichia coli* to intestinal epithelial cells. *J Appl Microbiol*, 2010; 106(3): 1013–21
- Roberts LC: The interaction of adherent invasive *Escherichia coli* with intestinal epithelial cells, *in vitro* derived M-cells and macrophages. *Acta Pharmacol Sin*, 2008; 31(3): 361–66
- Wang CT, Xie YH, Digestion DO: [Research of correlation between intestinal bacteria and digestive system diseases.] *Medical Recapitulate*, 2015 [in Chinese]
- Song M, Zhou L, Liu X et al: [The relationship between digestive system diseases complicated with osteoporosis and intestinal flora.] *Chinese Journal of Osteoporosis*, 2018 [in Chinese]
- Tyrer PC, Frizelle FA, Keenan JJ: *Escherichia coli*-derived outer membrane vesicles are genotoxic to human enterocyte-like cells. *Infect Agent Cancer*, 2014; 9(1): 2
- Murase K, Martin P, Porcheron G et al: Hlyf produced by extraintestinal pathogenic *Escherichia coli* is a virulence factor that regulates outer membrane vesicle biogenesis. *J Infect Dis*, 2016; 213(5): 856–65
- Riballo E, Kühne M, Rief N et al: A pathway of double-strand break rejoining dependent upon ATM, Artemis, and proteins locating to gamma-H2AX foci. *Molecular Cell*, 2004; 16(5): 715–24
- Chowdhury D, Keogh M C, Ishii H et al: γ -H2AX dephosphorylation by protein phosphatase 2A facilitates DNA double-strand break repair. *Mol Cell*, 2005; 20(5): 801–9
- Sayed AE, Igarashi K, Watanabeasaka T et al: Double strand break repair and γ -H2AX formation in erythrocytes of medaka (*Oryzias latipes*) after γ -irradiation. *Environ Pollut*, 2017; 224: 35–43
- Solier S, Sordet O, Kohn KW et al: Death receptor-induced activation of the Chk2- and histone H2AX-associated DNA damage response pathways. *Mol Cell Biol*, 2009; 29(1): 68–82
- Ivashkevich A, Redon CE, Nakamura AJ et al: Use of the γ -H2AX assay to monitor DNA damage and repair in translational cancer research. *Cancer Lett*, 2011; 327(1–2): 123–33
- Janaki DV, Nagarani N, Yokes BM et al: Genotoxic effects of profenofos on the marine fish, *Therapon jarbua*. *Toxicology Mech Methods*, 2012; 22(2): 111–17
- Lobb RJ, Becker M, Wen SW et al: Optimized exosome isolation protocol for cell culture supernatant and human plasma. *J Extracell Vesicles*, 2015; 4: 27031
- Momen-Heravi F, Balaj L, Alian S et al: Current methods for the isolation of extracellular vesicles. *Biol Chem*, 2013; 394(10): 1253–62
- Pienimaeki-Roemer A, Kuhlmann K et al: Lipidomic and proteomic characterization of platelet extracellular vesicle subfractions from senescent platelets. *Transfusion*, 2015; 55(3): 507–21
- Alvarez-Erviti L, Seow Y, Yin H et al: Delivery of siRNA to the brain by systemic injection of targeted exosomes. *Nat Biotechnol*, 2011; 29(4): 341–45
- Duthie SJ, Dobson VL: Dietary flavonoids protect human colonocyte DNA from oxidative attack *in vitro*. *Eur J Nutr*, 1999; 38(1): 28–34
- Follmann W, Birkner S: The use of cultured primary bovine colon epithelial cells as a screening model to detect genotoxic effects of heterocyclic aromatic amines in the comet assay. *J Toxicol Environ Health A*, 2008; 71(13–14): 947–53
- Robichová S, Slamenová D: Study of N-nitrosomorpholine-induced DNA strand breaks in Caco-2 cells by the classical and modified comet assay: influence of vitamins E and C. *Nutr Cancer*, 2001; 39(2): 267–72
- Anderson D, Hambly RJ, Yu TW et al: The effect of potassium diazoacetate on human peripheral lymphocytes, human adenocarcinoma colon caco-2 cells, and rat primary colon cells in the comet assay. *Teratog Carcinog Mutagen*, 2015; 19(2): 137–46
- Ferreira RF, Souza DR, Souza AS: Factors that induce DNA damage involving histone H2AX phosphorylation. *Radiology*, 2015; 277(1): 307–8
- Oh KS, Bustin M, Mazur SJ et al: UV-induced histone H2AX phosphorylation and DNA damage related proteins accumulate and persist in nucleotide excision repair-deficient XP-B cells. *DNA Repair*, 2011; 10(1): 5–15
- Solier S, Sordet O, Kohn KW et al: Death receptor-induced activation of the Chk2- and histone H2AX-associated DNA damage response pathways. *Mol Cell Biol*, 2009; 29(1): 68–82