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ORIGINAL ARTICLE

Evaluation of *Helicobacter pylori*-derived outer membrane vesicles on the expression of inflammatory cytokines

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ABSTRACT

Background and Objectives: *Helicobacter pylori* infection has been increasingly linked to extra-gastric diseases. Outer membrane vesicles are a key virulence factor of *H. pylori*. This study investigates the influence of *H. pylori*-derived outer membrane vesicles on inflammatory marker expression in human hepatoma cells (HepG2).

Materials and Methods: Outer membrane vesicles were isolated through ultracentrifugation and characterized using dynamic light scattering technique (DLS) and a Field Emission Scanning Electron Microscope (FE-SEM). Protein concentrations were measured via the Bradford assay. HepG2 cells treated with outer membrane vesicles were analyzed for IL-6, TNF-α, TLR-4, TGF-β, and PPAR-γ mRNA expression by RT-qPCR. Cell viability was assessed through an MTT assay. The prevalence of *H. pylori* virulence-associated genes (*babA2*, *sabA*, and *oipA*) was determined by PCR.

Results: The results showed a high prevalence of *sabA* (91.7%), *babA2* (75%), and *oipA* (66.7%). FE-SEM and DLS analyses confirmed the presence of bleb-shaped nanovesicles ranging in size from 50 to 450 nm. *H. pylori*-derived outer membrane vesicles significantly upregulated the expression of pro-inflammatory markers (TLR-4, PPAR-γ, TNF-α, and IL-6), while downregulating TGF-β expression.

Conclusion: These findings underscore the potential role of nanoparticles in driving inflammatory responses and influencing host cell signaling, which may play a key role in liver-related pathologies.

Keywords: *Helicobacter pylori*; Gene expression; Gastric cancer; Real-time polymerase chain reaction; Virulence factor

INTRODUCTION

Helicobacter pylori (*H. pylori*) is considered as a microaerophilic bacterium that serves as a spiral pathogen and infects nearly fifty percent of the global population (1-3). While it is widely recognized as the most common cause of chronic gastritis (CG), peptic ulcer disease (PUD), and gastric adenocarcinoma (4, 5), accumulating evidence suggests its involvement in diseases beyond the gastrointestinal system. These extra-gastric diseases include Parkinson's disease, type 2 diabetes, respiratory conditions, neurological disorders, cardiovascular diseases, anemia, idiopathic thrombocytopenic purpura, and Non-Alcoholic Fatty Liver Disease (NAFLD) (6-8). The potential mechanisms linking *H. pylori* infection to systemic diseases involve direct and indirect effects. Direct mechanisms may include the interaction of *H. pylori*

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or its components with host tissues, while indirect effects involve systemic inflammation, production of pro-inflammatory markers, biochemical mediators, and antibodies (9). The association between *H. pylori* and hepatobiliary diseases was first reported in 1998, when *H. pylori* DNA was detected in a Chilean patient with chronic cholecystitis (10). Since then, numerous surveys disclosed a possible role for *H. pylori* as a high-risk marker for liver cirrhosis, hepatocellular carcinoma, and fatty liver disease (FLD), particularly its non-alcoholic form (NAFLD) (10-13). In terms of virulence factors, outer membrane vesicles (OMVs) have gained significant attention. These nano-sized particles, released naturally by *H. pylori*, serve as carriers for toxins, adhesions, and other virulence markers, including VacA, CagA, BabA, and SabA (11, 12, 14).

Also, in this bacterium, three main adhesion factors are related to gastric cancer: sialic acid-binding adhesion A (SabA), outer inflammatory protein A (OipA), and blood group antigen-binding adhesion A (BabA). The results of studies indicated that the ability to discriminate was higher when three of the four antigens —SabA, OipA, and BabA—were used in combination (15). On the surface of gastric epithelial cells, BabA, by attaching to Lewis blood group receptors, participates in colonization. Furthermore, SabA binds to sialic acid-containing glycolconjugate domains, resulting in chronic inflammation and atrophy (16).

OMVs are crucial for bacterial survival and communication and profoundly influence host-pathogen interactions. These vesicles are related to immune modulation, delivering bacterial components directly into host cells and triggering inflammatory responses (9, 17-19). Recent studies indicate that OMVs may contribute to the progression of liver problems, including fibrosis and inflammation, by targeting hepatocytes (18-22). Hepatocytes, the primary parenchymal cell type of the liver, comprise over 80% of the liver's cellular population and are vital for maintaining homeostasis. They play key roles in immune regulation, tissue repair, and inflammatory responses (20). When exposed to bacterial components, such as OMVs, hepatocytes can release pro-inflammatory mediators and upregulate molecular markers, potentially initiating or exacerbating liver inflammation (19, 20). This process is facilitated by the intrinsic properties of OMVs, including their size, composition, and ability to carry virulence factors, allowing them to effectively interact with hepatocytes and modulate immune responses. This research aims to explore the impact of OMVs on inflammatory pathways in HepG2. By examining the expression of key inflammatory markers and assessing cellular responses, we aim to provide insights into the role of OMVs in liver-related pathologies. Understanding these mechanisms is critical for improving clinical management of *H. pylori*-associated extra-gastric diseases and refining therapeutic strategies.

MATERIALS AND METHODS

Sample collection. Gastric tissue biopsies were collected from 60 *H. pylori* positive patients during upper gastroduodenal endoscopy at Velayat Hospital, Qazvin, Iran. These 60 patients were selected from a total of 167 individuals presenting with digestive symptoms. At least two tissue biopsy specimens were obtained (the gastric antrum and the corpus regions) from each patient. Samples were transported on ice (4°C) in Fluid Thioglycolate Medium (Merck, Germany) to preserve viability. Bacterial urease activity was evaluated using a rapid urease test kit (I.P.K., Iran). Subsequently, the samples were transferred to the Clinical Microbiology Research Center in Qazvin for further analysis and processing.

Biopsy preparation, isolation of *H. pylori* strains and culture condition. Biopsy samples were homogenized using a glass rod under a Class II laminar flow hood to ensure sterility. Four to five loopfuls of the homogenized material were inoculated onto *H. pylori*-specific modified agar (Pronaisa, Spain) enriched with 7% (v/v) defibrinated sheep blood. The cultures were incubated at 37°C for five to seven days under microaerophilic conditions (N2: %85, CO2: %10 and O2: %5), by using an Anoxomat system (Mart Microbiology, Holland). The presence of *H. pylori* strains was confirmed by Gram staining and biochemical assays, including catalase, urease, and oxidase tests. Isolated strains were preserved in 250 µL of brain-heart infusion (BHI) broth (Merck, Germany) supplemented with 10% (v/v) Gibco-fetal bovine serum and 15% glycerol, and stored at -70°C for further use.

DNA extraction and quality assessment of *H. pylori* strains. After sub-culturing, single *H. pylori* colonies were selected, and lawn cultures were pre-

pared for DNA extraction using the WizPrep gDNA Mini Kit (Microbial, English), according to the kit instructions. The concentration and purity of the extracted deoxyribonucleic acid were analyzed using Thermo Scientific™ NanoDrop™ Micro volume spectrophotometry, and the 260/280 and 260/230 absorbance ratios were assessed to ensure high-quality DNA. The structural integrity of the DNA was further confirmed through electrophoresis on a 0.8% w/v (weight/volume) agarose gel. The extracted DNA was subsequently stored at -70°C for further processing.

Molecular genotyping of virulence factors of BabA2, SabA and OipA genes. A conventional PCR was done to detect the *babA2*, *sabA*, and *oipA* genes using a DNA thermal cycler (SimpliAmp Thermal Cycler PCR System, USA). The primers and the product sizes for each gene are presented in Table 1. Each reaction was performed in a total volume of 20 µL. comprising 10 µL Master Mix, 8 µL distilled water, 1 µL DNA template, and 1 µL of each forward and reverse primer. The PCR protocol started in initial denaturation at 94°C for 5 minutes, 30 cycles of denaturation at 94°C for 1 minutes, and annealing at specific temperatures (59°C for *babA2* and *sabA*, and 61°C for *oipA*) for 45 seconds, and extension at 72°C for 1 minute. A final extension phase was carried out at 72°C for 10 minutes. The amplified DNA fragments were analyzed using agarose gel electrophoresis. PCR products of expected lengths were separated on a 1% agarose gel, stained with a fluorescent DNA dye, and visualized under ultraviolet (UV) light using the Vilber GmbH imaging system (Germany).

Isolation of nanoparticles from selected strains liquid cultures. PCR analysis was conducted on 60 gastric biopsy specimens obtained from patients undergoing endoscopy for various gastric conditions. Only biopsies with histological evidence of *H. pylori* infection were included for bacterial culture and further analysis. In the subsequent phase, a subset of *H. pylori* strains (strains 1-5) were selected from these isolates for OMV extraction. The selected strains were sub-cultured in Brucella Media (BRU BROTH -Merck, Germany) supplemented with 5-10% (v/v) fetal calf serum (Gibco, USA) and incubated for two to three days at 37°C under a microaerophilic atmosphere, as mentioned above. Gentle shaking during incubation prevented bacterial clumping. OMVs were extracted from the bacterial supernatant following incubation. First, 500 mL of the culture medium was centrifuged at low speed (14,000 × g, 20 minutes, 4°C) to pellet the bacteria. The supernatant was then filtered through a sterile bottle-top filter (Corning, pore size: 0.45 µm, NY, USA) to remove debris. OMVs were isolated by ultracentrifugation (200,000 × g, 180 minutes, 4°C) using a Beckman Coulter ultracentrifuge (CA, USA) (21). The pelleted OMVs were rinsed twice with Gibco- phosphate-buffered saline solution (PBS, pH 7.2-7.4, Germany) and stored at -70°C until further analysis. OMV morphology and size were confirmed using a Field Emission Scanning Electron Microscope (FE-SEM) and dynamic light scattering technique (DLS). Protein concentrations of the OMVs were determined with a Bradford assay kit (Protocib, Iran) based on the absorbance shift of Coomassie Brilliant Blue (G-250) dye (CBBG), measured at 595 nm using a spectrophotometer.

Human hepatocytes cells culture. The HepG2 human liver carcinoma cell line was obtained from the Bon-Yakhte Institute (Iran). Cells were propagated in low-glucose Dulbecco's Modified Eagle Medium (DMEM; Sigma, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco), 1% penicillin–streptomycin (100 U/mL and 100 µg/mL;

Table 1. Oligonucleotide primers used for PCR in this research

Gene	Sequences	Annealing	Product	Reference
BabA	F: CCAAACGAAACAAAAAGCGT	59	105	This study
	R: GCTTGTGTAAAAGCCGTCGT			
SabA	F-CTCTCTCTCGCTTGCGGTAT	59	187	This study
	R-TTGAATGCTTTGCCTCAATG			
OipA	F: GTTTTTGATGCATGGGATTT	61	401	This study
	R: GTGCATCTCTTATGGCTTT			
16S rRNA	F: GCGCAATCAGCGTCAGGTAATG	53	522	(20)
	R: GCTAAGAGAGCAGCCTATGTCC			

Merck, Germany), and 2 mM L-glutamine. Cultures were incubated at 37°C under humidified conditions containing 5% CO_2 and 21% O_2. Once the monolayers reached approximately 70-80% confluence, they were detached using sterile trypsin–EDTA (Gibco, USA) and subcultured. Cell attachment was verified microscopically, and cultures were examined every 72 hours to assess growth and morphology.

Assessment of cell viability by the MTT assay. Cell viability of the HepG2 line was determined through a colorimetric MTT cytotoxicity assay using a commercial kit (Mitocib, Iran) based on the manufacturer's guidelines. In brief, HepG2 cells were seeded into 96-well flat-bottom microplates (clear polystyrene) at a density of 5×10^3 cells/cm². Once cultures reached approximately 70-80% confluence, they were exposed to varying concentrations (5, 10, 15, 20, and 25 µg/mL) of *H. pylori*-derived vesicles and incubated overnight. Following 24 hours of treatment, 10 µL of MTT reagent was added to each well, and the plates were further incubated for 4 hours at 37°C. Then, 100 µL of DMSO was introduced into each well to dissolve the resulting formazan crystals. The optical density (OD) was read at 570 nm with a reference wavelength of 630 nm using a microplate spectrophotometer (Eppendorf, Germany). The percentage of viable cells was determined using the formula:

Cell survival = $(X_{Treatment} - X_{Blank}) / (X_{Control} - X_{Blank}) \times 100\%$ (Where, "X" = Absorbance).

Cellular exposition to *H. pylori* -derived OMVs. HepG2 cells were cultured in 6-well plates at a seeding density of 5×10^5 cells/cm² and maintained for 24 hours in a humidified incubator under standard culture conditions (37°C, 21% O2, 5% CO_2). Once the cells reached approximately 70% to 90% confluence, they were rinsed three times with phosphate-buffered saline (PBS; Gibco, pH 7.2, Germany) and the medium was replaced with DMEM containing streptomycin/penicillin for another 24 hours. Subsequently, cells were exposed to OMVs (10 µg protein/mL) overnight. Untreated Hepatoma Cells served as the control group. All experimental setups, including OMV-treated and control conditions, were performed in duplicates and repeated three times to ensure reproducibility. After 24 hours of incubation, HepG2 cell pellets were collected for RNA extraction. The expression levels of anti-inflammatory and pro-inflammatory cytokines were subsequently quantified using precise and standardized molecular techniques.

RNA isolation and quantitative real-time PCR-method. Total RNA was extracted from HepG2 cells treated with *H. pylori*-derived nanoparticles and untreated control cells using the Ana-Cell Total RNA Extraction Kit (Ana Cell Tech, Iran), following the manufacturer's protocol. RNA concentration and purity were evaluated using a Thermo Scientific NanoDrop Microvolum ND-1000 spectrophotometer (USA), with purity assessed by the A260/A280 ratio. RNA integrity was confirmed through ribosomal RNA analysis via agarose gel electrophoresis. Extracted RNA was stored at -70°C until cDNA synthesis. First-strand cDNA synthesis was carried out using a commercial RTs enzyme (Reverse transcriptase) kit (Thermo Scientific, America) based on the instruction. Synthesized cDNA was stored at −20°C for the additional work (real-time PCR (RT-qPCR)) analysis. The expression levels of IL-6, TNF-α, TLR-4, TGF-β, and PPAR-γ were quantified using a Rotor-Gene Q real-time PCR system (Corbett RT-PCR Rotorgene 6000, Germany) and Biofact™ 2X Real-Time PCR Master Mix (South Korea). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control gene. Each qPCR mixture consisted of 10 µL of 2× SYBR Green Master Mix, 6 µL of nuclease-free water, 1 µL each of forward and reverse primers (total 2 µL), and 2 µL of cDNA template. The primer sequences and amplicon sizes are listed in Table 2. Amplification specificity was validated through melting curve analysis, and PCR products were additionally confirmed by agarose gel electrophoresis. All reactions were done in triplicate. Expression levels of target genes were assessed using the $2^{-}\Delta\Delta Ct$, with GAPDH serving as the normalization control.

Statistical analysis. Statistical evaluations were performed using SPSS software (version 22.0) and GraphPad Prism (version 8.1.1; La Jolla, CA, USA). Comparisons among experimental groups were conducted using a one-way ANOVA test. A significance threshold of $p < 0.05$ was applied. Levels of statistical significance were represented as P, *P, **P, ***P, and ****P, corresponding to $p < 0.05$, $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively. In line with previous literature, p-values below 0.05 were interpreted as statistically significant.

Table 2. Sequence of primers used for real time PCR studies

Gene	Primer Sequences	References
TGF-β	F: 5'-CCCAGCATCTGCAAAGCTC-3'	This study
	R: 5'-GTCAATGTACAGCTGCCGCA-3'	
PPAR	F: 5'-GCCGCCCTACAACGAGATCA-3'	This study
	R: 5'-CCACCAGCAGTCCGTCTTTGT-3	
TNF-alpha	F: :5′-CCG AGG CAG TCA GAT CAT CTT-3'	This study
	R:AGC TGC CCC TCA GCT TGA-3'	
IL6	F:5′-TGAACTCCTTCTCCACAAGCG-3	This study
	R: 5′-TCTGAAGAGGTGAGTGGCTGTC-3′	
TLR4	F: :5′-ATATTGACAGGAAACCCCATCCA-3'	This study
	R: :5′-AGAGAGATTGAGTAGGGGCATTT-3'	
GAPDH	F:5′- TGATTCTACCCACGGCAAGTT-3'	(22)
	R:5′- TGATGGGTTTCCCATTGATGA-3'	

RESULTS

Characteristics of *H. pylori*-infected patients and frequency of *H. pylori* virulence genotypes. From 60 gastric biopsy specimens obtained from urease-positive patients, *H. pylori* strains were successfully isolated. Patients' demographic and clinical data are presented in Table 3. Patients ranged in age from 22 to 83 years (mean ± SD: 47 ± 13.4 years) and in weight from 39 to 100 kg (mean ± SD: 69 ± 13.5 kg). Men constituted 51.7% (31/60) of the patients. Among the clinical strains, the primary presenting symptoms and conditions were as follows: peptic ulcer disease (41.7%, 25/60), chronic gastritis (28.3%, 17/60), gastroesophageal reflux (8.3%, 5/60), gastric cancer (3.3%, 2/60), dyspepsia (3.3%, 2/60), intestinal metaplasia (6.7%, 4/60), duodenal ulcer (5%, 3/60), and hiatus hernia (3.3%, 2/60). Patient medical histories included the following conditions: diabetes (3.3%, 2/60), non-gastrointestinal cancer (13.3%, 8/60), kidney dialysis (3.3%, 2/60), organ transplantation (3.3%, 2/60), drug addiction (3.3%, 2/60), COVID-19 positivity (1.7%, 1/60), neurological disease (1.7%, 1/60), convulsion (1.7%, 1/60), hepatitis B virus positivity (1.7%, 1/60), positive stool occult blood test (13.3%, 8/60), abdominal pain (75%, 45/60), antibiotic use in the last three months (15%, 9/60), alcohol use (3.3%, 2/60), smoking (15%, 9/60). Other conditions such as portal hypertensive gastropathy and anemia were present in 5% (3/60) (Table 4). There was no statistically significant association between age groups and *H. pylori* infection ($p > 0.05$). The prevalence of virulence genes *babA2*, *sabA*, and *oipA* was assessed via PCR. Among the 60 non-repetitive *H. pylori* strains, 75% (45/60) were positive for *babA2*, 91.7% (55/60) for *sabA*, and 66.7% (40/60) for *oipA* (Table 3).

Associations between virulence markers and clinical outcomes. These genotypes (*sabA*, *babA2*, and *oipA*) were significantly more prevalent among patients with peptic ulcer disease (100%, 92%, and 80%, respectively), duodenal ulcers (100%, 100%, and 66.6%), gastric cancer (100%, 100%, and 50%), gastroesophageal reflux (60%, 80%, and 60%), dyspepsia (100%, 50%, and 50%), and intestinal metaplasia (75%, 50%, and 25%). Notably, the *sabA* genotype was consistently observed in all patients with peptic ulcers, duodenal ulcers, gastric cancer, and hiatus hernia (100% prevalence). Conversely, the *babA2* and *oipA* genotypes showed no significant correlation with hiatus hernia.

Morphological characterization of OMVs secreted by *H. pylori*. OMVs secreted by five selected *H. pylori* strains were extracted and characterized. These five *H. pylori* strains were selected based on patient demographics, histopathology, gastrointestinal diseases diagnosed via endoscopy, and virulence factors (Table 5). The selected strains represented various clinical presentations: two were from patients with peptic ulcer disease, and a third was isolated from a patient with nausea, gastrointestinal bleeding, and pain, who was later diagnosed with gastric cancer. A fourth strain was isolated from an individual with duodenal ulcers, and the fifth strain was derived from a patient suffering from chronic gastritis and duodenal

Table 3. Demographic characteristics, frequency of virulence genotypes, and the relationship between each gene and clinical outcomes

Disease demographic	% (N)	BabA2 75% (45/60)	SabA 91.7% (55/60)	OipA 66.7% (40/60)
PUD	41.66% (25/60)	92% (23/25)	100% (25/25)	80% (20/25)
GER	8.3% (5/60)	80% (4/5)	60% (3/5)	60% (3/5)
IM	6.66% (4/60)	50% (2/4)	75% (3/4)	25% (1/4)
CG	28.3% (17/60)	47.05% (8/17)	88.2% (15/17)	70.5% (12/17)
DU	5% (3/60)	100% (3/3)	100% (3/3)	66.6% (2/3)
GC	3.33% (2/60)	100% (2/2)	100% (2/2)	50% (1/2)
HH	3.3% (2/60)	0% (0/2)	100% (2/2)	0% (0/2)
Dyspepsia	3.3% (2/60)	50% (1/2)	100% (2/2)	50% (1/2)

Peptic ulcer disease (PUD), Gastroesophageal reflux (GER), Intestinal metaplasia (IM), Chronic gastritis (CG), Duodenal ulcers (DU), Gastric cancer (GC), Hiatus hernia (HH).

Table 4. Demographic characteristics of 60 patients with *H. pylori*-positive

Clinical status	% (n=60)
Abdominal pain	75% (45/60)
Smoker	15% (9/60)
High weigh>80kg	20 (29.8%)
Alcoholic	3.3% (2/60)
Diabetes	3.3% (2/60)
Neurological disease positive	1.7% (1/60)
Convulsion	1.7% (1/60)
Kidney dialysis	3.3% (2/60)
Organ transplant recipient	3.3% (2/60)
Drug addiction	3.3% (2/60)
Hepatitis B virus positive	1.7% (1/60)
Hypertensive gastropathy and anemia	5% (3/60)
Antibiotic use in the last three months	15% (9/60)
Covid-19 positive	1.7% (1/60)
Occult blood test (OB)	13.33% (8/60)

ulcer. Morphological analysis using FE-SEM revealed double-layered, round vesicles ranging from <100 nm to >450 nm (Fig. 1). Dynamic light Scattering particle size distribution analysis (DLS) technique confirmed a vesicle size range of approximately 50-450 nm.

Evaluation of toxicity of *H. pylori* OMVs on HepG2 cells. The toxicity of *H. pylori*-derived OMVs on HepG2 cells was assessed using the MTT assay. Cells were cultured to 70-80% confluence before exposure to OMVs at concentrations ranging from 5 to 25 µg/mL. After 24 hours, a significant reduction in HepG2 cell viability was observed, with the most pronounced effects at concentrations of 5-25 µg/mL compared to control cells ($P < 0.05$) (Fig. 2). Based on these results, a concentration of 10 µg/mL was selected for subsequent cell culture experiments.

Impact of *H. pylori*-derived OMVs on inflammatory gene expression in HepG2 cells. The RT-qPCR technique was used to evaluate the expression of inflammatory markers (IL6, TNF-α, TLR-4, TGF-β, and PPAR-γ) in HepG2 cells exposed to OMVs from *H. pylori* strains (strain 1-5). The housekeeping gene GAPDH was used for normalization. As shown in Fig. 3, OMVs significantly upregulated the expression of IL6, TNF-α, TLR-4, and PPAR-γ, compared to control cells. In contrast, TGF-β expression was downregulated in response to OMVs. Strain 3 OMVs had the greatest effect on IL6, TNF-α, TLR-4, and PPAR-γ expression, whereas strain 2 OMVs downregulated IL6 and TNF-α. These findings highlight the potential of *H. pylori* OMVs to activate pro-inflammatory pathways in liver cells. Specifically, two out of three *sabA+babA2+oipA* strains (1 and 3) upregulated inflammatory factor expression, whereas the non-triple positive strains (2 and 4) demonstrated minimal pro-inflammatory properties. Strain 2 did not harbor SabA, and OMVs showed a decrease in IL6 and TNF-α levels. Also, strain 4 did not harbor OipA, and OMVs showed a decrease in TLR-4 and PPAR-γ expression.

Exposure of HepG2 cells to OMVs derived from various *H. pylori* strains resulted in a significant upregulation of IL-6, TNF-α, TLR4, and PPAR-γ mRNA

Table 5. Characteristics of clinical *H. pylori* strains used in this study.

	Strain number				
Demographic characteristics and virulence genotypes	**Strain 1**	**Strain 2**	**Strain 3**	**Strain 4**	**Strain 5**
Disease outcome	PUD	PUD	GC	DU	CG
Sex (F/M)	M	F	M	F	M
Age/year	26	31	50	72	57
Weight/kg	52	88	60	70	62
OipA	+	+	+	-	+
SabA	+	-	+	+	+
BabA2	+	+	+	+	+
SabA+ BabA2	+	-	+	+	+
SabA+ OipA	+	-	+	+	+
BabA2+ OipA	+	+	+	+	+
SabA+ BabA2+ OipA	+	-	+	-	+

PUD: peptic ulcer disease; CG: chronic gastritis; DU: Duodenal ulcer; GC: Gastric cancer; M: Male; F: Female.

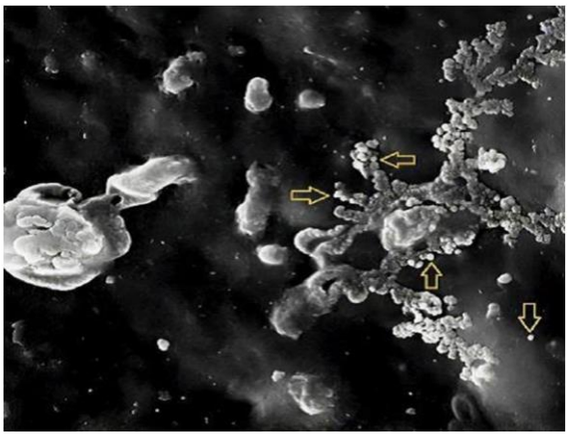

Fig. 1. Morphological analysis of OMVs derived from *H. pylori* was performed. FE-SEM imaging revealed that the vesicles were spherical, exhibited a bilayer structure, and varied in size ranging from 50 to 450 nm.

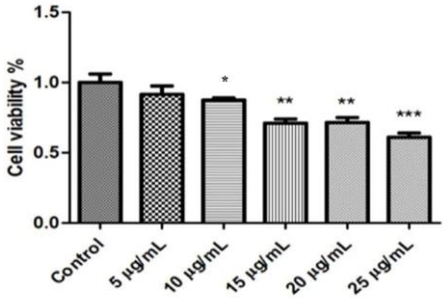

Fig. 2. Impact of different concentrations of *H. pylori*-derived OMVs (5- 25 µg/mL) on the cell viability of HEP-G2 cells

levels. Conversely, TGF-β transcript levels were reduced following treatment with OMVs from the same strains. Results are presented as mean ± standard error of the mean (SEM) from three independent experiments. Statistical significance was assessed using one-way ANOVA with post hoc analysis, and indicated as *P, **P, ***P, and ****P corresponding to $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively.

DISCUSSION

For over three decades, *H. pylori* has been recognized as a primary associated with a variety of gastrointestinal conditions, including chronic gastritis, peptic ulcer disease, and gastric cancer. Recent research, however, suggests that its role may extend beyond these traditional disorders, including cardiovascular disorders, hematologic conditions, and, more notably, liver-related problems such as NAFLD, obesity, hepatic fibrosis, and even hepatocellular carcinoma (7). These associations suggest that the impact of *H. pylori* may extend beyond the stomach, affecting organs like the liver (Hepatoma Cells) through the release of virulence factors that may be transported via various routes, including OMVs (23-26). OMVs, small proteoliposomes naturally secreted by both pathogenic and non-pathogenic Gram-negative bacteria, including *H. pylori*, have gained attention in recent years for their role in pathogenesis and immune modulation.

It should be noted that OMVs consist of bacterial

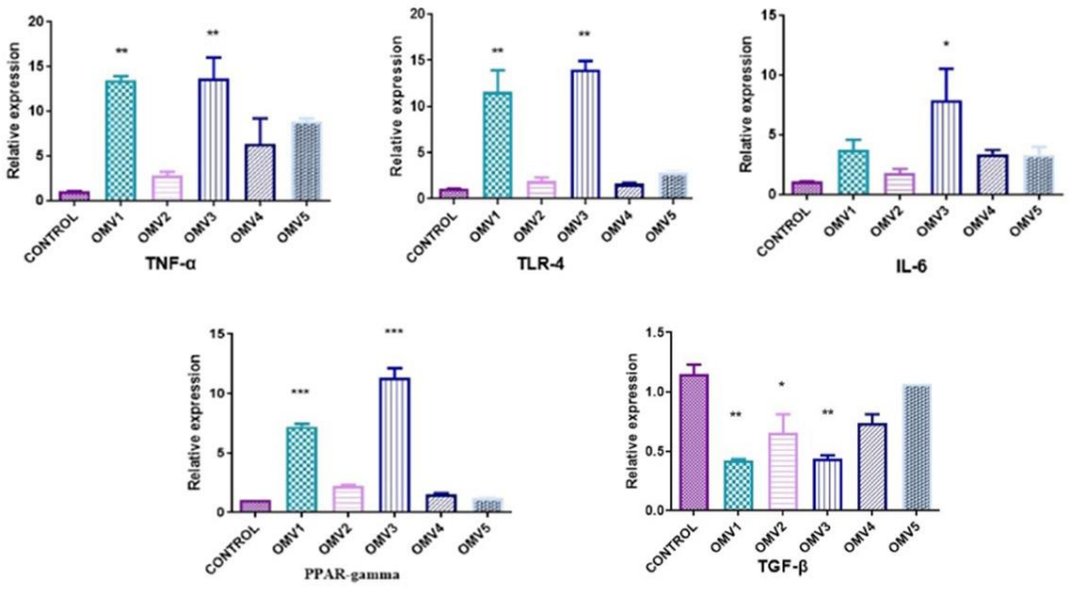

Fig. 3. Gene expression analysis of liver inflammatory markers in HepG2 cells treated with 10 µg/mL of *H. pylori* OMVs.

components such as LPS, bacterial outer membrane, membrane proteins, membrane lipids, and peptidoglycans. Among these, LPS is the most important component of the OMVs, and it can harbor some intracellular molecules such as DNA, RNA, intracellular proteins, and enzymes, most of which are PAMPs that can activate the immune responses in hosts (27, 28).

These vesicles, due to their nanoparticle size, can travel through host tissues, cross epithelial barriers, and deliver bacterial components such as lipopolysaccharides (LPS), proteins, RNA, and DNA to immune cells, including hepatocytes. Such characteristics make OMVs potent carriers of bacterial virulence factors, and their interaction with host cells is a key area of interest in understanding *H. pylori*'s role in liver diseases (29-31). In this context, inflammation has been recognized as a central mechanism driving liver disease progression. The liver is a highly immune-sensitive organ that plays a critical role in metabolic regulation and immune defense. It is especially susceptible to endotoxins such as LPS, which can directly cause liver damage (19). Previous studies have indicated that *H. pylori* infection and its associated OMVs are capable of inducing inflammatory responses in various cell types, including hepatic stellate cells (HSCs), which are involved in liver fibrosis. *H. pylori* OMVs have been shown to activate these cells and upregulate markers of liver fibrosis, such as α-SMA, TIMP-1, vimentin, and β-catenin. (32-34). Additionally, these OMVs have been found to influence autophagy markers and alter hepatocyte exosome content, further supporting their role in liver pathology (7, 35). The present study specifically aimed to explore the effects of *H. pylori* OMVs on inflammatory pathways in hepatocytes. We focused on the expression of some pro-inflammatory markers, such as IL-6, TNF-α, TLR-4, and PPAR-γ, which are known to play pivotal roles in the immune response and liver homeostasis. Our findings showed that exposure of HepG2 cells to *H. pylori* OMVs resulted in increasing in the expression levels of mRNA in these inflammatory markers.

IL-6, in particular, was notably upregulated, which is consistent with its known role as a key cytokine involved in immune defense, acute-phase response, and hepatocyte homeostasis. IL-6 signals hepatocytes to produce acute-phase proteins during immune responses, thus contributing to both inflammation and tissue repair (36). The upregulation of IL-6 in response to *H. pylori* OMVs is in line with previous studies, including Yildirim et al.'s work, which showed elevated IL-6 levels in patients with *H. pylori* infection. Moreover, Tsai et al. identified *H. pylori* NAP protein as a major virulence factor that also contributes to the induction of IL-6 expression (37, 38). Similarly, we observed increased expression of TNF-α in HepG2 cells following OMV

treatment, corroborating findings by Polyzos et al., who reported significantly higher levels of TNF-α in *H. pylori*-positive patients compared to controls. The involvement of TNF-α in liver damage is well-established, and its overexpression is known to exacerbate liver injury, contributing to the progression of liver diseases (39). Another study further emphasized the significance of IL-6 and TNF-α in end-stage liver disease, suggesting that targeting these cytokines could be an effective therapeutic strategy in treating *H. pylori*-associated liver conditions (40). In a study conducted by Abdel-Razik et al. *H. pylori* infection was related to the level of pro-inflammatory cytokines. Also, after treatment of patients, reduction in pro-inflammatory markers was observed. Furthermore, the results indicated an increased risk of developing NAFLD, as measured by the NAFLD liver fat score (NAFLD-LFS). Overall, our findings are consistent with previous studies, demonstrating that *H. pylori*-derived nanoparticles induce IL6 gene expression in HepG2 cells (41). Similar to our results, Noori et al. evaluated the expression of Alzheimer's disease (AD)-associated genes and inflammatory markers in individuals with peptic ulcer disease (PUD) who were also *H. pylori*-positive. Results showed that *H. pylori* induced TLR-4 and TNF-α gene expression in brain cell lines (U87MG and 1321N1). The results from cells treated with *H. pylori* demonstrated a potential contribution of the infection to the incidence and development of (AD) (42). Additionally, our study demonstrated that *H. pylori*-derived OMVs significantly modulate the expression of TGF-β, a key regulator in liver fibrosis and carcinogenesis. TGF-β is known to be involved in all stages of liver problems progression, from the early phase injury through inflammation and fibrosis to the eventual development of cancer (43, 44). Our findings are consistent with another study that linked *H. pylori* infection to hepatic fibrosis and modulated TGF-β expression in liver tissue (45). This suggests that *H. pylori*-derived nanoparticles (OMVs) may not only contribute to inflammatory responses but also play a role in the fibrotic process and progression to liver cancer. Moreover, the study revealed that *H. pylori* nanoparticles influenced the expression of PPAR-γ in hepatocytes. PPAR-γ is known to be involved in the regulation of inflammation and metabolic processes, and its upregulation in response to *H. pylori* infection has been associated with gastric cancer. Konturek et al. showed that *H. pylori*-positive gastric cancer patients had significantly higher levels of PPAR-γ expression, which was reduced following *H. pylori* eradication (46). These data further support the idea that *H. pylori* infection and its OMVs play a crucial role in modulating inflammatory pathways and could potentially contribute to the development of hepatocellular carcinoma.

Also, our study provides evidence that *H. pylori*-derived OMVs strongly interact with hepatocytes, triggering pro-inflammatory responses and upregulating key markers such as IL-6, TNF-α, TLR-4, and PPAR-γ. These findings underscore the potential of *H. pylori* vesicles (OMVs) in liver-related disorders, particularly those involving chronic inflammation, fibrosis, and cancer. Additional works are needed to explore the precise mechanisms by which *H. pylori* OMVs influence liver disease progression and their potential as therapeutic targets in managing *H. pylori*-associated liver conditions.

CONCLUSION

H. pylori-derived OMVs are rich in various biologically active components, such as porins, membrane proteins, adhesions, and other molecules that can modulate immune responses. These vesicles, released during *H. pylori* infection, act as carriers for virulence factors and can migrate from the gastric environment through the gastrointestinal tract and into the bloodstream. This suggests that OMVs has a vital role in *H. pylori* pathogenesis. They interact directly or indirectly with immune cells, stimulating the release of cytokines and contributing to tissue damage. Our findings showed that the presence of the *sabA* gene in *H. pylori* is related to an increased risk of duodenal ulcers, peptic ulcer disease, dyspepsia, gastric cancer, and hiatus hernia in adult patients with *H. pylori* infection in Iran. These results provide valuable insights for physicians to improve early diagnosis and treatment strategies for patients at higher risk of these conditions. Multiple studies showed a connection between *H. pylori*-derived OMVs and extra-gastric diseases, particularly liver diseases. However, the exact mechanisms and extent of this association remain unclear due to limited data. Our study underscores the significant impact of *H. pylori*-derived OMVs on HepG2 cells, particularly in inducing pro-inflammatory responses. While this study did not involve animal models or human

subjects, which limits its ability to fully validate the findings, it provides a solid foundation for further research. Future studies should focus on exploring additional cytokines, confirming these findings in clinical trials, and further investigating the role of *H. pylori*-derived OMVs in liver disorders.

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REFERENCES

1. Moosazadeh Moghaddam M, Bolouri S, Golmohammadi R, Fasihi-Ramandi M, Heiat M, Mirnejad R. Targeted delivery of a short antimicrobial peptide (CM11) against *Helicobacter pylori* gastric infection using concanavalin A-coated chitosan nanoparticles. *J Mater Sci Mater Med* 2023; 34: 44.
2. Lormohammadi L, Nikkhahi F, Bolori S, Karami AA, Hajian S, Rad N, et al. High level of resistance to metronidazole and clarithromycin among *Helicobacter pylori* clinical isolates in Qazvin province, Iran. *Gene Rep* 2022; 26: 101494.
3. Reshetnyak VI, Burmistrov AI, Maev IV. *Helicobacter pylori*: Commensal, symbiont or pathogen? *World J Gastroenterol* 2021; 27: 545-560.
4. Kim J, Wang TC. *Helicobacter pylori* and gastric cancer. *Gastrointest Endosc Clin N Am* 2021; 31: 451-465.
5. Huang Y, Wang Q-l, Cheng D-d, Xu W-t, Lu N-h. Adhesion and invasion of gastric mucosa epithelial cells by *Helicobacter pylori*. *Front Cell Infect Microbiol* 2016; 6: 159.
6. Pellicano R, Ianiro G, Fagoonee S, Settanni CR, Gasbarrini A. Extragastric diseases and *Helicobacter pylori*. *Helicobacter* 2020; 25 Suppl 1: e12741.
7. Mohammadi M, Attar A, Mohammadbeigi M, Peymani A, Bolori S, Fardsanei F. The possible role of *Helicobacter pylori* in liver diseases. *Arch Microbiol* 2023; 205: 281.
8. Tamura A, Fujioka T, Nasu M. Relation of *Helicobacter pylori* infection to plasma vitamin B12, folic acid, and homocysteine levels in patients who underwent diagnostic coronary arteriography. *Am J Gastroenterol* 2002; 97: 861-866.
9. He J, Liu Y, Ouyang Q, Li R, Li J, Chen W, et al. Helicobacter pylori and unignorable extragastric diseases: Mechanism and implications. *Front Microbiol* 2022; 13: 972777.
10. Rabelo-Gonçalves EMA, Roesler B, Zeitune J (2014). *Helicobacter pylori* and Liver–Detection of Bacteria in liver Tissue from patients with Hepatocellular carcinoma using Laser Capture Microdissection Technique (LCM). Trends in *Helicobacter pylori* Infection.
11. Mekonnen HD, Fisseha H, Getinet T, Tekle F, Galle PR. *Helicobacter pylori* infection as a risk factor for hepatocellular carcinoma: a case-control study in ethiopia. *Int J Hepatol* 2018; 2018: 1941728.
12. Xu G, Ma S, Dong L, Mendez-Sanchez N, Li H, Qi X. Relationship of *Helicobacter pylori* infection with nonalcoholic Fatty Liver Disease: A meta-analysis. *Can J Gastroenterol Hepatol* 2023; 2023: 5521239.
13. Chen X, Peng R, Peng D, Xiao J, Liu D, Li R. An update: is there a relationship between *H. pylori* infection and nonalcoholic fatty liver disease? why is this subject of interest? *Front Cell Infect Microbiol* 2023; 13: 1282956.
14. Turner L, Bitto NJ, Steer DL, Lo C, D'Costa K, Ramm G, et al. *Helicobacter pylori* outer membrane vesicle size determines their mechanisms of host cell entry and protein content. *Front Immunol* 2018; 9: 1466.
15. Chew Y, Chung H-Y, Lin P-Y, Wu D-C, Huang S-K, Kao M-C. Outer membrane vesicle production by *Helicobacter pylori* represents an approach for the delivery of virulence factors caga, vaca and urea into human gastric adenocarcinoma (Ags) cells. *Int J Mol Sci* 2021; 22: 3942.
16. Mommersteeg MC, Yu J, Peppelenbosch MP, Fuhler GM. Genetic host factors in *Helicobacter pylori*-induced carcinogenesis: Emerging new paradigms. *Biochim Biophys Acta Rev Cancer* 2018; 1869: 42-52.
17. Taubenheim C (2019). The genomic content of outer membrane vesicles (OMVs) in freshwater cyanobacteria.
18. Jarzab M, Posselt G, Meisner-Kober N, Wessler S. *Helicobacter pylori*-derived outer membrane vesicles (OMVs): role in bacterial pathogenesis? *Microorganisms* 2020; 8: 1328.
19. Gong J, Tu W, Liu J, Tian D. Hepatocytes: A key role in liver inflammation. *Front Immunol* 2023; 13: 1083780.
20. Schulze RJ, Schott MB, Casey CA, Tuma PL, McNiven MA. The cell biology of the hepatocyte: A membrane trafficking machine. *J Cell Biol* 2019; 218: 2096-2112.
21. Melo J, Pinto V, Fernandes T, Malheiro AR, Osório H, Figueiredo C, et al. Isolation method and characterization of outer membranes vesicles of *Helicobacter pylori* grown in a chemically defined medium. *Front Microbiol* 2021; 12: 654193.
22. Zhang T, Liu C-F, Zhang T-N, Wen R, Song W-L. Overexpression of peroxisome proliferator-activated receptor γ coactivator 1-α protects cardiomyocytes from li-

popolysaccharide-induced mitochondrial damage and apoptosis. *Inflammation* 2020; 43: 1806-1820.
23. Amano A, Takeuchi H, Furuta N. Outer membrane vesicles function as offensive weapons in host–parasite interactions. *Microbes Infect* 2010; 12: 791-798.
24. Ait-Ahmed Y, Lafdil F. Novel insights into the impact of liver inflammatory responses on primary liver cancer development. *Liver Res* 2023; 7: 26-34.
25. Chmiela M, Kupcinskas J. Pathogenesis of *Helicobacter pylori* infection. *Helicobacter* 2019; 24 Suppl 1(Suppl Suppl 1): e12638.
26. Liu Y, Li D, Liu Y, Shuai P. Association between *Helicobacter pylori* infection and non-alcoholic fatty liver disease, hepatic adipose deposition and stiffness in Southwest China. *Front Med (Lausanne)* 2021; 8: 764472.
27. Wang S, Gao J, Wang Z. Outer membrane vesicles for vaccination and targeted drug delivery. *Wiley Interdiscip Rev Nanomed Nanobiotechnol* 2019; 11(2): e1523.
28. Yue Y, Xu J, Li Y, Cheng K, Feng Q, Ma X, et al. Antigen-bearing outer membrane vesicles as tumour vaccines produced in situ by ingested genetically engineered bacteria. *Nat Biomed Eng* 2022; 6: 898-909.
29. Nilsson H-O, Mulchandani R, Tranberg K-G, Stenram U, Wadström T. *Helicobacter* species identified in liver from patients with cholangiocarcinoma and hepatocellular carcinoma. *Gastroenterology* 2001; 120: 323-324.
30. Zoaiter M, Nasser R, Hage-Sleiman R, Abdel-Sater F, Badran B, Zeaiter Z. *Helicobacter pylori* outer membrane vesicles induce expression and secretion of oncostatin M in AGS gastric cancer cells. *Braz J Microbiol* 2021; 52: 1057-1066.
31. Furuyama N, Sircili MP. Outer membrane vesicles (OMVs) produced by gram-negative bacteria: structure, functions, biogenesis, and vaccine application. *Biomed Res Int* 2021; 2021: 1490732.
32. Zahmatkesh ME, Jahanbakhsh M, Hoseini N, Shegefti S, Peymani A, Dabin H, et al. Effects of exosomes derived from *Helicobacter pylori* outer membrane vesicle-infected hepatocytes on hepatic stellate cell activation and liver fibrosis induction. *Front Cell Infect Microbiol* 2022; 12: 857570.
33. Bolori S, Shegefti S, Baghaei K, Yadegar A, Moon K-M, Foster LJ, et al. The Effects of *Helicobacter pylori*-Derived outer Membrane Vesicles on hepatic stellate cell activation and liver Fibrosis In Vitro. *Biomed Res Int* 2023; 2023: 4848643.
34. Shegefti S, Bolori S, Nabavi-Rad A, Dabiri H, Yadegar A, Baghaei K. *Helicobacter pylori*-derived outer membrane vesicles suppress liver autophagy: A novel mechanism for *H. pylori*-mediated hepatic disorder. *Microb Pathog* 2023; 183: 106319.
35. Chen X, Peng R, Peng D, Xiao J, Liu D, Li R. An update: is there a relationship between *H. pylori* infection and nonalcoholic fatty liver disease? why is this subject of interest? *Front Cell Infect Microbiol* 2023; 13: 1282956.
36. Sun Y, Gu J, Liu R, Zhou H, Lu L, Dai X, et al. IL-2/IL-6 ratio correlates with liver function and recovery in acute liver injury patients. *APMIS* 2019; 127: 468-474.
37. Yildirim Z, Bozkurt B, Ozol D, Armutcu F, Akgedik R, Karamanli H, et al. Increased exhaled 8-isoprostane and interleukin-6 in patients with *Helicobacter pylori* infection. *Helicobacter* 2016; 21: 389-394.
38. Tsai C-C, Kuo T-Y, Hong Z-W, Yeh Y-C, Shih K-S, Du S-Y, et al. *Helicobacter pylori* neutrophil-activating protein induces release of histamine and interleukin-6 through G protein-mediated MAPKs and PI3K/Akt pathways in HMC-1 cells. *Virulence* 2015; 6: 755-765.
39. Polyzos SA, Kountouras J, Papatheodorou A, Patsiaoura K, Katsiki E, Zafeiriadou E, et al. *Helicobacter pylori* infection in patients with nonalcoholic fatty liver disease. *Metabolism* 2013; 62: 121-126.
40. Ponzetto A, Figura N. Interleukin 6 predicts mortality in patients with End-Stage Liver Disease. *Clin Gastroenterol Hepatol* 2018; 16: 783.
41. Abdel-Razik A, Mousa N, Shabana W, Refaey M, Elhelaly R, Elzehery R, et al. *Helicobacter pylori* and non-alcoholic fatty liver disease: A new enigma? *Helicobacter* 2018; 23(6): e12537.
42. Noori M, Mahboobi R, Nabavi-Rad A, Jamshidizadeh S, Fakharian F, Yadegar A, et al. *Helicobacter pylori* infection contributes to the expression of Alzheimer's disease-associated risk factors and neuroinflammation [Heliyon 9(9) (September 2023) e19607]. *Heliyon* 2023; 9(9): e19944.
43. Fabregat I, Moreno-Càceres J, Sánchez A, Dooley S, Dewidar B, Giannelli G, et al. TGF-β signalling and liver disease. *FEBS J* 2016; 283: 2219-2232.
44. Dooley S, ten Dijke P. TGF-β in progression of liver disease. *Cell Tissue Res* 2012; 347: 245-256.
45. Goo M-J, Ki M-R, Lee H-R, Yang H-J, Yuan D-W, Hong I-H, et al. *Helicobacter pylori* promotes hepatic fibrosis in the animal model. *Lab Invest* 2009; 89: 1291-1303.
46. Konturek PC, Kania J, Kukharsky V, Raithel M, Ocker M, Rembiasz K, et al. Implication of peroxisome proliferator-activated receptor γ and proinflammatory cytokines in gastric carcinogenesis: link to *Helicobacter pylori*-infection. *J Pharmacol Sci* 2004; 96: 134-143.