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RESEARCH ARTICLE

Engineered extracellular vesicles coated with an antimicrobial peptide for advanced control of bacterial sepsis

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Abstract

Alarming sepsis-related mortality rates present significant challenges to healthcare services globally. Despite advances made in the field, there is still an urgent need to develop innovative approaches that could improve survival rates and reduce the overall cost of treatment for sepsis patients. Therefore, this study aimed to develop a novel multifunctional therapeutic agent for advanced control of bacterial sepsis. Extracellular vesicles (EVs) isolated from lipopolysaccharide (LPS) induced HepG2 (hepatocellular carcinoma cells) (iEV) displayed an average particle size of 171.63 ± 2.77 nm, a poly dispersion index (PDI) of 0.32 ± 0.0, and a zeta potential (ZP) of −11.87 ± 0.18 mV. Compared to HepG2 EV, LPS induction significantly increases the EV protein concentration, PDI and ZP, reduces the average size and promotes cell proliferation and cytoprotective effects of the isolated EVs (iEVs) against LPS-induced cytotoxicity. Coating of iEV with a cationic antimicrobial peptide (AMP) to form PC-iEV slightly changed their physical properties and shifted their surface charge toward neutral values. This modification improved the antibacterial activity (2-fold lower minimum bactericidal concentration [MBC] values) and biocompatibility of the conjugated peptide while maintaining iEV cytoprotective and anti-inflammatory activities. Our findings indicate the superior anti-inflammatory and antibacterial dual activity of PC-iEV against pathogens associated with sepsis.

KEYWORDS
antimicrobial peptides: extracellular vesicles, sepsis, therapeutics

1 | INTRODUCTION

Sepsis, a leading cause of death globally and in Sub-Saharan Africa, is characterised by a dysregulated immune response to infections, resulting in potentially fatal organ failure and potential mortality (Kiya et al., 2023; Wiersinga & van der Poll, 2022). Its current definition emphasises the non-homeostatic host response to infection. Despite the current treatment strategies recommended by the Surviving Sepsis Campaign (Lamas, 2022; Vincent, 2022), persistent infections account for a significant percentage (70%–80%) of sepsis-induced deaths due to widespread antimicrobial (including antibiotic) resistance and ineffective treatment choices (Liang & Yan, 2023). In addition, the pathophysiology and clinical manifestations of sepsis in patients are heterogeneous and complex (Wang & Liu, 2023), necessitating biomarkers for patient subgrouping to benefit from targeted and personalised therapies. Currently, available approaches for sepsis management and control mainly focus on the timely delivery of intravenous antibiotics to suppress the infection. Additionally, different anti-inflammatory medications, antioxidants and immunomodulators are administered to minimise inflammatory harmful reactions, along with supportive care to assist in stabilising critical

organs and systems. Despite of advances made in this field, there is still an urgent need to develop innovative strategies that could lead to development of multifunctional novel biomaterials with antibacterial, anti-inflammatory and cytoprotective activity (against bacterial toxins-induced cytotoxicity). These biomaterials could serve as effective antisepsis therapeutics, increasing survival rates and minimising the financial burden and total cost of treatment for sepsis patients.

EV-based therapeutics, particularly those generated from mesenchymal stem/stromal cells (MSCs) and immune cells, have demonstrated significant therapeutic potential against various diseases and pathological conditions (Abdel-Aal et al., 2019; Gürbay et al., 2005; Yadav & Talwar, 2019; Zandi et al., 2017). EV-based therapeutics have been derived from different types of cells, including MSCs, platelets, immune and epithelial cells (Abdel-Rahman et al., 2021; Ahadi et al., 2020; Chrzanowska et al., 2020; Esfandiari Mazandaran et al., 2019; Sharma et al., 2010), and blood samples from individuals with various pathological conditions (Kassab & Gedawy, 2018; Rautio et al., 2008; Stella et al., 2007). Importantly, EVs have inherently excellent delivery properties that make them an attractive candidate for therapeutic and drug delivery purposes. They have high cellular uptake rates, advanced biocompatibility, circulation clearance, prolonged circulation time in comparison to other nanosystems, and scalable ex vivo capacity. Additionally, EVs can be modified on their surface to improve their targeting capabilities and physical stability. Another advantage of EVs is their ability to cross biological barriers such as the blood-brain barrier and lung tissues, allowing them to reach tissues that may be difficult to access by other delivery methods (Rautio et al., 2008; Stella et al., 2007; Torchilin, 2006; Zaro, 2015). These properties make EVs a promising tool for therapeutic delivery in various diseases and conditions.

There is growing evidence of the efficacy of EV-based therapeutics and delivery systems in preclinical studies using various animal models of diseases (Fasiku et al., 2020). Furthermore, MSC EV-based therapeutics have been extensively studied (Hao et al., 2018; M. Hasanin, Taha, et al., 2022; M. S. Hasanin, El-Sakhawy, et al., 2022; Omolo et al., 2021; Pawełczyk et al., 2018; Ray et al., 2019; Turabee et al., 2017) and presented as a safe alternative to stem cell-based therapy, which comes with the added risk of tumorigenicity, immune rejection and thrombogenicity (Hoang et al., 2022). EV-based therapeutics have also demonstrated beneficial effects in a variety of pathological conditions, including cardiovascular, respiratory and neurological disorders. Similarly, exosomes (EVs, subtype) isolated from sepsis patients, in vitro and in vivo models of sepsis, LPS-induced macrophages, and LPS-induced cancer cells have shown an immunomodulatory activity that could alleviate sepsis's hyperinflammatory or immunosuppression phases via different mechanisms of action (Abdel-Aal et al., 2019; Gürbay et al., 2005; Li et al., 2022; Sharma et al., 2010; Yadav & Talwar, 2019; Zandi et al., 2017). Moreover, some EV-based candidates have moved toward the clinical trial stages (Curcio et al., 2020; Devnarain et al., 2021), demonstrating the translational value of these promising characteristic properties of EV-based therapeutics.

Recently, advanced drug delivery approaches such as surface modification and functionalisation (Taresco et al., 2018), exosome mimics (Abou-Yousef et al., 2021; Lapčík et al., 1998), exosomal cargo replacement (Schaefer & Schaefer, 2009) and multifunctional (Lapčík et al., 1998) and stimuli-responsive coating (Bayer, 2020) have been widely used to improve the efficacy of EV-based therapeutics against various diseases including cancer (Baek et al., 2022; Behzadi et al., 2017). Despite the advances of the modified EVs, research to reduce unpredicted EV-related side effects and enhance the efficacy of EV-based therapeutics remains an active research area. Advanced drug delivery approaches have been used to enhance the ability of therapeutic EVs for tissue targeting to improve their clinical efficacy and reduce undesired side effects (Bayer, 2020; Cho, 2020). Among these approaches, the impact of surface modification strategies on EV-based therapeutics has been extensively studied (Gudbergsson et al., 2019).

On the other hand, antimicrobial peptides (AMPs) are gaining attention as a promising antibiotic therapy alternative for bacterial infections and sepsis due to their multiple mechanisms of action (Kumar et al., 2018), strong efficacy, minimal drug residue and ease of production and modification (Yang et al., 2021). However, AMPs have limitations in terms of bioavailability, stability and tolerability (Teixeira et al., 2020). Therefore, research directed towards enhancing their effectiveness and safety profile is of critical necessity. One potential approach to address these limitations of AMPs involves the utilisation of nano-delivery systems (Radaic et al., 2020). Various strategies, such as self-assembly (Lei et al., 2018; Tan et al., 2022), nanoencapsulation (Hassan et al., 2021; Saúde et al., 2014) and surface conjugation (Fan et al., 2015; Rai et al., 2016) onto various metallic and organic nanoparticles have been explored for the delivery of AMPs against bacterial sepsis. We previously reported the identification of the novel cationic AMP (abbreviated as AMP-A in this study), which has the amino acid sequence (RKKKKLLRKKC), using a computer-aided design approach. AMP-A is a lysine-rich AMP with an excellent bacterial pore-forming ability, as proposed by the computational studies done. AMP-A has shown good antibacterial and biocompatibility properties, and it has been co-delivered with another antibacterial agent (hydrogen peroxide) via a chitosan-based hydrogel for topical wound healing purposes (Fasiku et al., 2021). Hence, coating the negatively charged EVs with such cationic AMP could enhance AMPs stability and systemic bioavailability. Therefore, this approach could serve as an effective strategy to address the limitations associated with AMPs systemic administration and enhance their effect in the management of sepsis.

Several studies have documented the surface modification of EVs with different types of peptides, such as cell-penetrating peptides (Huang et al., 2023; Nakase, 2021; Xu et al., 2021), myocardium-targeting peptides (Wang et al., 2018), tumour-targeting peptides (Tran et al., 2023; Zhou et al., 2023), brain-targeting peptides (Li et al., 2023) and anti-inflammatory peptides (Kim et al., 2021) for management of different disease categories. However, there have been no reports on utilising EVs to deliver AMPs either through encapsulation or surface modification for any disease condition. Therefore, using engineered EV with

intrinsic anti-inflammatory and cytoprotective activity to deliver therapeutic molecules, such as AMPs, would be an innovative and cutting-edge approach to combating sepsis. Applying such advanced drug delivery and discovery approaches can enhance the efficacy of EV-based therapeutics against sepsis and provide an advanced treatment option to reduce the burden of this disease.

Sepsis-related EVs (EVs isolated from sepsis patients and sepsis mice) have shown promising therapeutic activity against sepsis. These EVs have improved survival rates and prevent tissue damage in animal models by promoting differentiation, migration and proliferation of T-lymphocytes (Bhagwan Valjee et al., 2023). Furthermore, EVs isolated from LPS-induced melanoma cancer cells (an in vitro sepsis model) have demonstrated a prominent antisepsis activity through improving endothelial functions and enhancing immune response, thereby preventing sepsis-associated tissue damage and reducing mortality rate in sepsis model mice (Li et al., 2022). Accordingly, we hypothesise that EVs isolation from LPS-induced HepG2 cancer cells would have similar cytoprotective and immunomodulatory properties.

Therefore, this study aimed to investigate the anti-inflammatory and cytoprotective activity of LPS-induced HepG2 EVs (iEVs) against LPS-induced inflammation and cytotoxicity, as well as explore their potential to improve the delivery and stability of cationic AMPs and to present this peptide coated EV (PC-iEVs) as novel multifunctional antisepsis EV-based therapeutic agent with advanced antibacterial, anti-inflammatory and cytoprotective activity. The data resulting from isolation, characterisation, cytoprotective evaluation of iEVs, peptide coating optimisation, and in vitro characterisation of PC-iEVs (antibacterial activity against *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*), cytoprotective and anti-inflammatory properties) are herein reported.

2 | MATERIALS AND METHODS

2.1 | Materials

Dulbecco's Modified Eagle Medium (DMEM) cell culture media and related materials were purchased from Whitehead Scientific (Lethabong, South Africa) and Gibco, Thermo-Fisher (USA). Highveld Biologicals (Johannesburg, South Africa) supplied us with cell lines used in this study. LPS from *E. coli* O111:B4 and MTT salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Merck Chemicals (Darmstadt, Germany). The total exosome isolation kit (from cell culture medium) was bought from Invitrogen Thermo-Fisher (USA). For the antibacterial activity studies, all reagents and materials were purchased from Biolab (Midrand, South Africa). Both bacterial strains (*S. aureus* ATCC 25923 and *E. coli* ATCC 25922 (O6-antigen)) were bought from DLD Scientific (Durban, South Africa). Purified distilled water (DW) used in this study was generated using a mill-Q water purification system (Merck, Germany). AMP-A (RKKKKLLRKKC) was synthesised by China Peptides Co. Ltd. (China), and ELISA kits for IL-6 and TNF-α analyses were purchased from ABclonal Technology (Wuhan, Hubie, China).

2.2 | Cell culture and isolation of HepG2 iEVs

HepG2 cells were cultured in 75 cm flasks using DMEM, supplemented with foetal bovine serum, glutamate and antibiotic, and incubated in 5% carbon dioxide humidified atmosphere at 37°C till 80% confluency. After that, the cell culture medium was replaced with serum-free DMEM (for control EVs) or serum-free DMEM containing 10 μg/mL LPS (for LPS-induced EVs) and incubated at the same conditions for 24 h. The media was then collected to isolate EVs using an Invitrogen total exosomes isolation kit (from cell culture medium), as per the manufacturer's instructions. In brief, the collected medium was centrifuged at 2000 × *g* for 20 min, and the supernatant was transferred carefully to a new collection tube. Then, 15 mL of the cell-free culture medium was gently mixed with 7.5 mL of exosomes isolation reagent and incubated overnight at 4°C and, after that, centrifuged at 10,000 × *g* for 60 min at 4°C temperature. The supernatant was carefully aspirated and discarded, while EV pellets were resuspended with a suitable volume of 1X phosphate-buffered saline (PBS) for further investigation.

2.3 | Characterisation of isolated EVs (iEVs)

2.3.1 | Particle size, poly dispersion index (PDI) and surface charge

EVs and coated EVs were characterised using dynamic light scattering techniques to determine their average hydrodynamic diameter, PDI and zeta potential (ZP). In brief, a Zetasizer Nano ZS90 (Malvern Instruments, UK) device set at 633 nm laser and 173 detection optics was used to measure the parameters mentioned above of EVs at room temperature (25°C).

2.3.2 | Morphology

Appropriate dilutions of EVs and coated EVs dispersions in PBS (0.1 M) were negatively stained using 2% uranyl acetate solution after being placed onto a continuous nickel grid. The samples were left to dry at room temperature, and finally, images of the EVs were taken using a transmission electron microscope (TEM, JEOL JEM 1400) (JEOL, Peabody, MA, USA).

2.3.3 | Protein quantification

The EV protein content was quantified using a Nanodrop 2000 bioanalyser spectrophotometer using a previously reported method (Alvarez et al., 2012). Briefly, the absorbance of EV dispersion at 280 nm was measured, and the quantity of EV protein was estimated using human albumin as standard.

2.4 | The effect of iEVs on cell viability of HepG2 and HEK293 Cell lines

The effect of both HepG2 EVs and iEVs on cell viability was evaluated using a previously reported MTT assay method and two types of cell lines: human embryonic kidney HEK 293 and HepG2. Briefly, both cell lines were incubated in suitable environments using DMED as a cell culture media till 80% confluency. Then, cells were seeded (seeding density = 20.000 cells/well) into a sterile 96-well plate, incubated for 24 h, and then treated with different concentrations of both types of EVs (10, 7.5, 5 and 2.5 μg/mL of EV protein) in DMEM for 24 h. Control cells were treated with DMEM only. After that, 120 μL/well MTT solution was added after aspiration of treatments. The formed formazan crystals were dissolved by replacing the media with 100 μL/well Sterile DMSO 4 h later, and absorbances were recorded using a microplate spectrophotometer (Spectrostar Nano, Germany) at 570 nm after 1 h of DMSO addition. Cell viability was calculated as a percentage of control untreated cells. All treatments in this study were done in triplicate.

2.5 | Cytoprotective activity of iEVs against LPS-induced cytotoxicity

A previously reported method was implemented to determine the in vitro cytoprotective activity of iEVs against LPS-induced cytotoxicity. This method involved using a cell line model of cells that are affected by LPS (Hepatocellular carcinoma cell [HepG2]) and an MTT assay as an evaluation technique (Mosmann, 1983; Nishio et al., 2013; Pierdomenico et al., 2023). In brief, HepG2 cells were cultured as previously described in Section 2.2 until reaching 80% confluency, then were seeded in a 96-well plate (25,000 cells/well) and incubated for 24 h. The cells were then treated with a different set of treatments, including 200 μL of DMEM (Control untreated cells), 200 μL of 0.9 mg/mL of LPS in DMEM (LPS treated cells), 200 μL of 0.9 mg/mL of LPS and different concentrations of HepG2 EVs and iEVs (10, 7.5, 5 and 2.5 μg/mL of EVs protein) in DMEM. After 24 h of treatment, the MTT assay proceeded as described in Section 2.5.

2.6 | Preparation and optimisation of AMP-A-coated EVs (PC-iEVs)

AMP-A has a proven biocompatibility and antibacterial activity against both gram-negative and gram-positive bacteria and hence was used as a model of AMPs for coating purposes (Fasiku et al., 2021). Different amounts of AMP-A (3, 2 and 1 mg) were completely dissolved in 2 mL of purified DW and then added dropwise to an equivalent volume of EVs dispersion in PBS (0.1 mg/mL EVs protein) under continuous stirring at 300 rpm speed at room temperature for 1 h. Thereafter, DLS measurements and TEM imaging were used to confirm the successful coating with peptide through surface charge change and doubled layered morphology of the PC-iEVs, respectively, following the same procedure mentioned in Sections 2.3.1 and 2.3.2.

2.7 | Cytoprotective activity of PC-iEVs against LPS-induced cytotoxicity

The in vitro cytoprotective activity of PC-iEVs against LPS-induced cytotoxicity was evaluated using the same procedure used to evaluate the cytoprotective activity of iEVs described in Section 2.5. Different sets of treatments were made, including DMEM (Control untreated cells), 0.9 mg/mL of LPS in DMEM (LPS treated cells), 0.9 mg/mL of LPS with 10 μg/mL EV protein of iEVs, 0.9 mg/mL of LPS with 10 μg/mL EV protein of PC-iEVs and 150 μg/mL of peptide in DMEM. The EVs levels were chosen according to the results obtained from Sections 2.4 and 2.5, where we tested the effect of EVs and iEVs on HepG2 cell viability without

TABLE 1 Average particle size, PDI, ZP and protein concentration of HepG2 EVs and iEVs.

	Average particle size (nm)	PDI	ZP (mV)	Protein concentration (μg/mL)
HepG2 EVs	216.77 ± 4.63	0.12 ± 0.05	−15.13 ± 0.98	100 ± 4
HepG2 iEVs	171.63 ± 2.77	0.32 ± 0.01	−11.87 ± 0.18	120 ± 10

Note: Results were represented as mean ± standard deviation. The significance of differences was calculated using an unpaired *t*-test, $n = 3$.

and with LPS, respectively, while the peptide concentration was chosen to be equal to the corresponding peptide concentration in the optimised formula that contained the highest EVs concentration tested.

2.8 | Anti-inflammatory activity of PC-iEVs against LPS-induced inflammatory responses

The anti-inflammatory activity of C-iEVs was evaluated using a previously reported model (LPS-stimulated-HepG2 cells) (Pierdomenico et al., 2023). Briefly, IL-6 and TNF-α levels were measured using ELISA kits (AB clonal Technology, Wuhan, Hubie, China) in CCM after HepG2 cells were stimulated with 1 μg/mL LPS solution in DMEM for 12 h both with and without treatments. After being cultivated and plated in accordance with the instructions in the preceding Section 2.2, HepG2 cells were subjected to the following treatments (200 μL/well of each): DMEM (Control untreated), 1 μg/mL of LPS in DMEM (LPS-treated); 1 μg/mL of LPS and 0.01 mg/mL iEVs in DMEM; 1 μg/mL of LPS and 0.01 mg/mL PC-iEVs in DMEM; and 1 μg/mL of LPS and 0.15 mg/mL of peptide. ELISA kits were used as per the manufacturer's recommendations.

2.9 | Antibacterial activity of PC-iEVs

The effect of EV delivery of the AMP-A on its in vitro antibacterial activity was evaluated through determination of the minimum bactericidal concentration (MBC). Both AMP-A and PC-iEVs were examined against *E. coli* and *S. aureus* using a previously reported microdilution method (Jorgensen & Turnidge, 2015). Briefly, 5×10^5 colony-forming units per ml (CFU/mL) bacterial suspensions were prepared from an overnight culture of *S. aureus* and *E. coli*. Fifteen microliters of 5×10^5 CFU/mL bacterial suspension was added to each well in a 96-well plate that held 135 μL/well of successive dilutions (1:1) of the investigated substances (AMP-A, iEVs and PC-iEVs) in Mueller Hinton Broth. The concentrations of the coated exosomes were calculated as follows:

$$\text{PC} - \text{iEVs Concentration} = \frac{\text{AMP weight in mg} + \text{iEV weight in mg}}{\text{total volume of formulation in mL}}$$

The 96-well plate was then placed in a shaking incubator set to 100 rpm and 37°C for 24 h. Following this, 5 μL from each well was spotted on Muller–Hinton agar plates, and after the agar plates had been incubated for 24 h at 37°C, the MBC values were determined using naked-eye visual detection of no colonies growing.

2.10 | Statistical analysis

For statistical analysis, GraphPad Prism 8.4.3 (GraphPad Software Inc., USA) was utilised. Every experiment was conducted in triplicate, and the results were reported as mean ± standard deviation. Data were subjected to one-way ANOVA or *t*-test to verify statistical significance and were considered statistically significant at p-value <0.05.

3 | RESULTS AND DISCUSSION

3.1 | EVs characterisation

EVs isolated from the HepG2 cell line showed an average hydrodynamic size of 216.77 ± 4.63 nm, PDI of 0.12 ± 0.05 and ZP of −15.13 ± 0.98 mV as per DLS results (Table 1). LPS induction (10 μg/mL) of HepG2 cells promotes EV release. It significantly increases the EV protein concentration, PDI and ZP and reduces the average hydrodynamic size of the iEVs, as shown in Table 1. Similar results have been reported for the effect of LPS induction on EVs isolated from a murine alveolar macrophage cell line (Sui et al., 2021) and endothelial progenitor cells through a toll-like receptor-dependant mechanism (Xia et al., 2022).

HepG2 iEVs TEM images showed spherical shape morphology (Figure 1) when compared with control HepG2 iEVs that

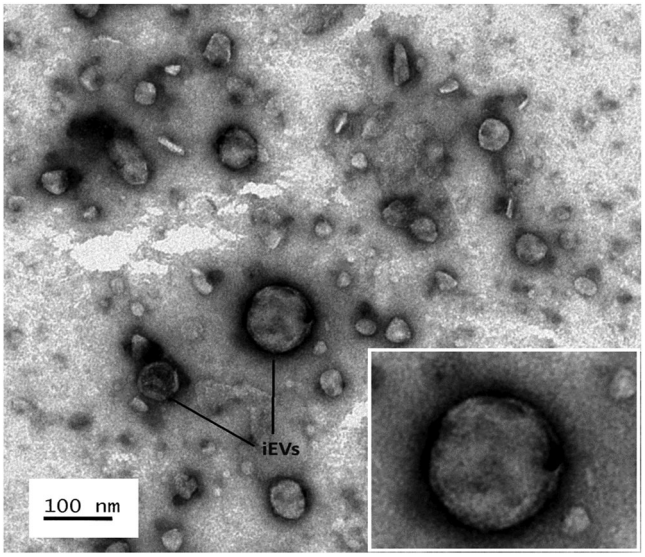

FIGURE 1 TEM images for HepG2 iEVs isolated using the precipitation method (magnified at the bottom right). The image also illustrated lipoprotein aggregates co-isolated with EVs (white circular particles). Scale bar: 100 nm.

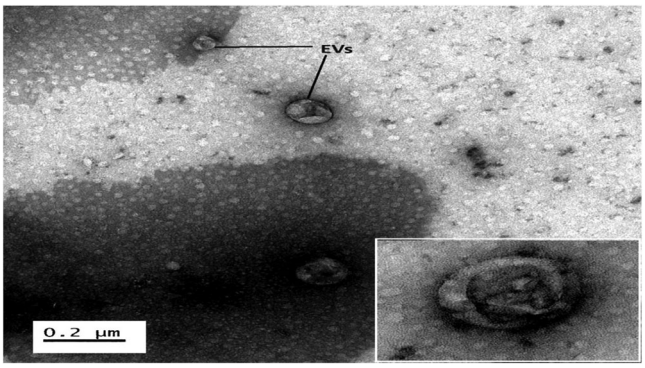

FIGURE 2 TEM images for HepG2 EVs isolated using the precipitation method (magnified in the bottom right). The image also illustrated lipoprotein aggregates co-isolated with EVs (white circular particles). Scale bar: 0.2 µm.

showed oval shape morphology, as presented in Figure 2.

3.2 | The effect of iEVs EVs on cell viability of HepG2 and HEK293 cell lines

The MTT assay was used to evaluate the effect of LPS-induced HepG2 EVs on cell proliferation of HepG2 and HEK293 Cells. As shown in Figure 3, EVs isolated from HepG2 cells (both EVs and iEVs) revealed increases in HepG2 cell viability within the concentration range used in this study while maintaining the cell viability of HEK 293 cell with a significant increase in the cell viability recorded for the highest concentration of iEVs (10 µg/mL EV protein). HepG2 iEVs-treated groups have shown a significantly higher HepG2 cell viability percentage than their respective HepG2 EVs-treated groups. Previously reported results for extracellular vesicles (EVs) isolated from non-small cell lung cancer have shown their ability to promote cell proliferation and inhibit cell apoptosis of both cancerous and non-cancerous cells through delivering alpha-smooth muscle actin protein (Huang et al., 2019). A similar molecular mechanism might be involved in the cytoprotective effect of HepG2 EVs.

3.3 | Cytoprotective activity of iEVs against LPS-induced cytotoxicity

LPS is a bacterial toxin that could induce inflammation in a variety of medical disorders, including sepsis. It is crucial for triggering an immunological reaction that can lead to cytotoxicity and malfunction of several organs (Nedeva, 2021). Furthermore,

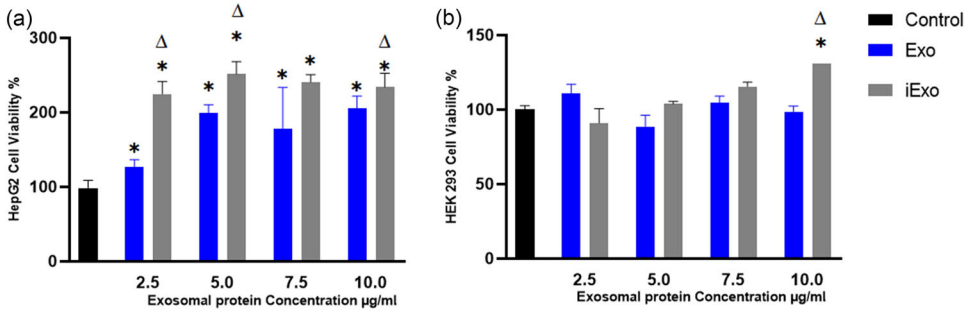

FIGURE 3 The effect of HepG2 EVs and LPS-induced HepG2 EVs in the cell viability of (a) HepG2 cell line and (b) HEK 293 Cells. $^{*}p < 0.05$ versus control (untreated cells), $^{\#}p < 0.05$ versus LPS 0.9 mg/mL, $^{\Delta}p < 0.05$ versus respective concentration of HepG2 EVs. There were significant increases in cell viability of LPS-induced HepG2 EVs-treated groups compared to HepG2 EVs-treated groups. Results were represented as mean ± standard deviation. The significance of differences was calculated using one-way ANOVA test, $n = 3$.

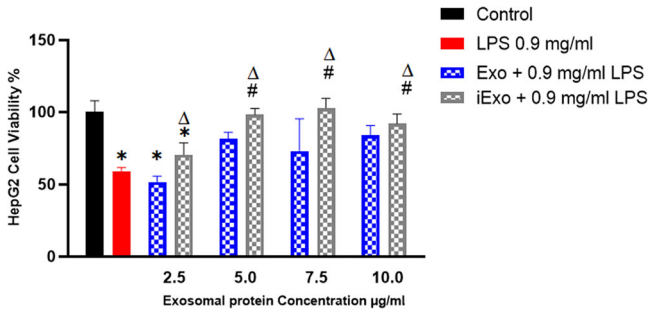

FIGURE 4 Cytoprotective effect of LPS-induced HepG2 EVs against LPS-induced cytotoxicity on HepG2 cell line. $^{*}p < 0.05$ versus control (untreated cells), $^{\#}p < 0.05$ versus LPS 0.9 mg/mL, $^{\Delta}p < 0.05$ versus respective concentration of HepG2 EVs. Results were represented as mean ± standard deviation. The significance of differences was calculated using one-way ANOVA test, $n = 3$.

in sepsis, LPS can have cytotoxic effects on numerous cells and organs (Rossol et al., 2011). Therefore, evaluation of cytoprotective activity against LPS-induced cytotoxicity and inflammatory responses is considered an essential parameter in developing new antisepsis therapeutics (Lehmann et al., 2014). In this study, a previously reported in vitro method for the evaluation of the cytoprotective activity of therapeutic agents against LPS-induced cytotoxicity has been used to evaluate the cytoprotective activity of iEVs. Figure 4 shows that LPS treatment (0.9 mg/mL) reduced the viability of HepG2 cells by approximately 50%, while coadministration of both EVs and iEVs with different concentrations attenuated the LPS cytotoxic effect. A dose-dependent cytoprotective effect was observed for HepG2 iEVs, with significant increases in cell viability compared to the respective concentrations of HepG2 EVs. Therefore, we used iEVs for delivery of the AMP-A due to their superior cytoprotective activity compared to control EVs. While LPS-induced and sepsis patient's EV have been linked with their therapeutic effect against sepsis and cytokine storm (Bhagwan Valjee et al., 2023; Li et al., 2022), this is the first report that evaluated the in vitro cytoprotective effect of LPS-induced HepG2 EVs against LPS-induced cytotoxicity.

3.4 | Preparation and optimisation of PC-iEVs

Recently, EVs have been introduced as advanced drug delivery carriers for AMPs (Deng et al., 2024); therefore, in this study, we used iEVs as a carrier for AMPs to confer the obtained system both antibacterial activity and cytoprotective activity against LPS-induced cytotoxicity and improved stability and antibacterial activity of the delivered peptide. Negatively charged iEVs were successfully coated with the positively charged AMP-A, as shown in the TEM images in Figure 5, through electrostatic interaction. Shifting of the negative surface charge of iEVs toward neutral values infer the success of the coating process; a similar trend has been reported before for the coating process of niosomes (Osman et al., 2024). For optimisation purposes, the effect of EV protein: peptide mass ratio on coating efficiency, average particle size, PDI and ZP of the coated iEVs is presented in Table 2. The optimum mass ratio with an almost neutral surface charge was found to be 1:15 (EV protein: peptide), which showed optimum characteristics of 202.03 nm average particle size, 0.11 PDI and 0.95 mV ZP. Furthermore, the double-layered structure of PC-iEVs (Figure 5) and the surface charge switching from −11.87 to +0.95 mV accompanied by a significant increase in the average particle size (from 176 to 202 nm) with significant reduction in PDI (from 0.32 to 0.11) could be attributed to

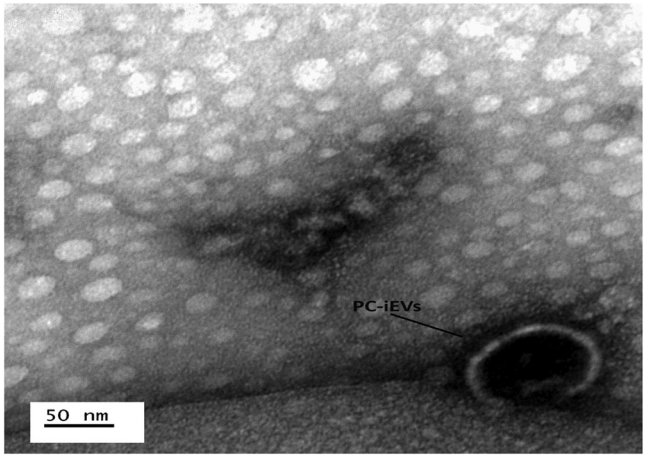

FIGURE 5 TEM images for PC-iEVs. The image also illustrated lipoprotein aggregates co-isolated with EVs (white circular particles). Scale bar: 50 nm.

TABLE 2 Optimisation of EV coating process showing the effect of mass ratio on average particle size, PDI, and zeta potential of PC-iEVs. Results were represented as mean ± standard deviation. The significance of differences was calculated using one-way ANOVA test, $n = 3$.

EV protein: Peptide mass ratio	Average particle size (nm)	PDI	ZP (mV)
1:0 (Uncoated EVs)	171.63 ± 2.77	0.32 ± 0.01	−11.87 ± 0.18
1:7.5	216.10 ± 7.23	0.30 ± 0.00	5.00 ± 1.20
1:10	293.53 ± 9.12	0.25 ± 0.02	9.74 ± 2.78
1:15	202.03 ± 0.60	0.11 ± 0.01	0.95 ± 1.42

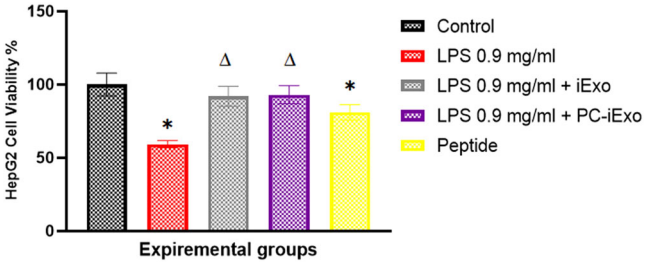

FIGURE 6 Cytoprotective effect of PC-iEVs against LPS-induced cytotoxicity and the effect of the AMP-A on cell viability of HepG2 cell line. $^{*}p < 0.05$ versus control (untreated cells), $^{\Delta}p < 0.05$ versus LPS 0.9 mg/mL. Results were represented as mean ± standard deviation. The significance of differences was calculated using a one-way ANOVA test, $n = 3$.

the successful coating with the AMP-A. The particles mono dispersion (PDI = 0.11) and small average particle size (202 nm) indicated the lower possibility of EVs agglomeration.

3.5 | Cytoprotective activity of PC-iEVs against LPS-induced cytotoxicity

Figure 6 shows that peptide coating did not affect the cytoprotective activity of iEVs against LPS-induced cytotoxicity, which proposes that EV cargo contents are responsible for their cytoprotective action (Deng et al., 2024). On the other hand, peptide anchoring on the EV surface improved its biocompatibility and reduced the peptide's cytotoxic effect against HepG2 cells. Improving the biocompatibility of therapeutic peptides through incorporation into nanosystems has been reported previously for peptides nanosystems (Piktel et al., 2019; Wei & Ma, 2022). Therefore, PC-iEVs might be used as a therapeutic agent to stop sepsis-related cell damage and improve the overall therapeutic outcomes of AMPs.

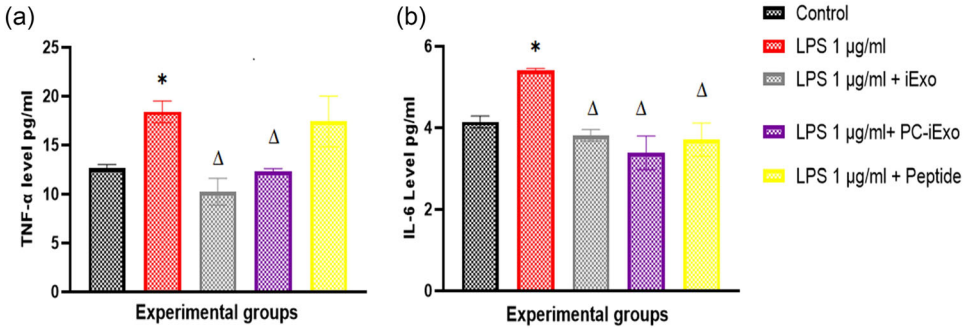

FIGURE 7 Anti-inflammatory effect of PC-iEVs against LPS-induced inflammatory responses (a) TNF levels, (b) IL-6 level among different treated groups. $^{*}p < 0.05$ versus Control (untreated cells), $^{\Delta}p < 0.05$ versus LPS 0.9 mg/mL. Results were represented as mean ± standard deviation. The significance of differences was calculated using one-way ANOVA test, $n = 3$.

TABLE 3 MBC results (mg/mL) of the HepG2 iEVs, PC-iEVs and AMP-A against *E. coli* and *S. aureus* after 24 h of incubation ($n = 3$).

	E. coli	*S. aureus*
iEVs	NA	NA
AMP-A	1.500	0.046
PC-iEVs	0.375	0.046

Abbreviation: NA, no activity.

3.6 | Anti-inflammatory activity of PC-iEVs against LPS-induced inflammatory responses

LPS could elicit an immunological response and can lead to the production of many cytokines, including IL-6 and TNF-α (Castellheim et al., 2009). Thus, using the HepG2 cell line (Mosmann, 1983; Pierdomenico et al., 2023), we explored the potential anti-inflammatory properties of PC-iEVs against LPS-induced inflammatory responses through the determination of IL-6 and TNF-α levels after PC-iEVs treatment. LPS (1 μg/mL) significantly increased the level of both inflammatory cytokines (IL-6 and TNF-α), as presented in Figure 7. While both iEVs and PC-iEVs treatments diminished the LPS-induced cytokines release with approximately equal values, peptide treatment only reduced LPS-induced IL-6 release and did not affect the LPS-induced TNF-α release, which may relate to the slight HepG2 cell viability reduction caused by peptide treatment. These findings promote iEVs and PC-iEVs as promising candidates for controlling the inflammatory responses mediated by IL-6 and TNF-α release in sepsis and other immune-related disorders.

3.7 | Antibacterial activity of PC-iEVs

The impact of iEVs delivery on the AMP-A's antibacterial effectiveness against both gram-negative (*E. coli*) and gram-positive (*S. aureus*) strains was assessed using a microdilution technique to determine the MBC. iEVs, within the concentration range used in this study, did not show any antibacterial activity against both strains. Table 3 demonstrates that, in comparison to bare AMP-A, PC-iEVs retained similar antibacterial activity against S. *aureus* and significantly improved activity against *E. coli* (2-fold lower MBC value). This enhancement of activity against gram-negative bacteria may be related to iEVs neutralising activity against the gram-negative outer membrane main component and virulent factor LPS through direct binding of lipid A of LPS and the lipid bilayer of EVs (Huang & Zhou, 2023; Kumari et al., 2023), which affecting their outer membrane integrity (Erridge et al., 2002). These results demonstrate iEVs potential to deliver AMPs, improving their antibacterial activity and cytocompatibility.

4 | CONCLUSION

In conclusion, this study reported a novel approach that aimed to develop an EV-based antisepsis therapeutic agent. This approach involved the investigation of the cytoprotective and anti-inflammatory properties of HepG2 iEVs and exploring their potential to deliver cationic AMPs through the coating process. EVs were successfully isolated and characterised before and after LPS induction. LPS stimulation-induced EV release from HepG2 cells affected EV's physical properties and improved cell proliferative and cytoprotective activity against LPS-induced cytotoxicity of the iEVs. Coating of iEVs with cationic AMP-A

significantly increased the peptide's antibacterial activity against *E. coli* (an LPS-producing bacteria). Moreover, it enhanced its cytocompatibility while not affecting the cytoprotective and anti-inflammatory activity of iEVs against LPS-induced response. The main limitation of this study was using a precipitation-based isolation technique, which isolates more than one subtype of EVs and affects the purity of the iEVs due to co-isolation of lipoprotein and lipoprotein aggregates (as shown in the TEM images), which also affects the using of protein concentration as a robust measurable parameter to evaluate and compare the therapeutic activity of iEVs. Therefore, further investigations that involve more accurate isolation and purification techniques, advanced EV molecular markers characterisation and robust measurable parameters to evaluate the therapeutic activity of iEVs are highly recommended. Our results suggest that PC-iEVs have the potential to be an effective, safe antisepsis EV-based therapeutic agent with advanced antibacterial, anti-inflammatory and cytoprotective activities.

AUTHOR CONTRIBUTIONS

Usri H. Ibrahim: Conceptualization; data curation; formal analysis; investigation; methodology; writing—original draft; writing—review and editing. **Mohammed A. Gafar**: Formal analysis; investigation; methodology; writing—review and editing. **Rene Khan**: Investigation; methodology; resources. **Abdelrahman Tageldin**: Investigation; methodology. **Thirumala Govender**: Methodology; resources; writing—review and editing. **Irene Mackraj**: Methodology. resources; writing—review and editing; supervision

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CONFLICTS OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article, and raw data will be available upon request from the corresponding Author.

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