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Curcumin nanoemulsion suppresses HPV oncogenes and inhibits cervical cancer progression: *in vitro* and *in vivo* study

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Abstract

Background Cervical cancer represents a major global health problem, ranking as the fourth most prevalent cancer among women across the globe. The primary risk factor associated with cervical intraepithelial neoplasia and cervical cancer is the human papillomavirus (HPV). Curcumin (Cur), extracted from the root of the Curcuma longa plant, is an anticancer, chemoprotective, and gene/protein regulating agent, which refers to its ability to exert beneficial effects in various aspects of cancer prevention and treatment.

Objectives This study investigated the tumor inhibitory effect (anti-tumoral effect) of a novel curcumin nanoemulsion (Cur-NE) on HPV^{+} TC-1 cells in vitro and in vivo.

Methods The MTT assay was used to evaluate the cytotoxicity of Cur-NE and Cur on TC-1 cancer cells and MC3T3 normal cells. In vitro assessment was performed using flow cytometry (Annexin/PI) to examine apoptosis and quantitative PCR (qPCR) analysis to determine the gene expression levels of *E6* and *E7* human papillomavirus oncogenes, as well as their associated protein factors, *p53* and *Rb*. In addition, C57BL/6 female mice burdening HPV + TC-1 tumor as cervical cancer models were used to investigate the tumor inhibitory effect of the Cur-NE in vivo compared to free curcumin.

Results *In vitro* anti-tumoral studies showed that apoptosis and inhibiting cellular proliferation in TC-1 cells were induced effectively by curcumin nanoemulsion. Accordingly, curcumin nanoemulsion reduced mRNA expression levels of *E6* and *E7* HPV oncogenes and increased *p53* and *Rb* levels in a concentration lower than free curcumin ($P < 0.05$). Furthermore, the suppression and inhibition of subcutaneous TC-1 tumor growth were more pronounced with the curcumin nanoemulsion compared to free curcumin ($P < 0.01$).

Conclusion These preeminent preclinical results indicate the potential of this curcumin nanoformulation as an efficient treatment approach for cervical cancer.

Keywords Cervical cancer, HPV, Curcumin, Nanoemulsion, TC-1 cells, Anti-tumor

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Introduction

Cervical cancer, ranked as the fourth most prevalent malignancy affecting women globally, contributes to approximately 600,000 new cases and 340,000 deaths annually [1]. Although numerous factors such as smoking, immunosuppression, suboptimal sexual health, and non-participation in screening exist to elevate the risk of cervical cancer, human papillomavirus (HPV) infection emerges as a prominent causal factor contributing to nearly all cases [2]. Over the past three decades, extensive research on HPV has led to the identification of several low-risk and high-risk types associated with prevalent forms of cervical cancer [3]. Among these, HPV types 16 and 18 are recognized as the most high-risk types, accounting for over 70% of cervical cancer cases [4]. Based on studies, *E6* and *E7*, the two prominent oncogenes of HPV, play a central role [5]. These viral proteins are responsible for inactivating the critical tumor suppressor proteins p53 and retinoblastoma protein (Rb), thus enabling unchecked cell cycle progression and promoting cancerous transformation [6–8].

E6 and E7 oncoproteins target and degrade the tumor suppressor proteins p53 and Rb, respectively [9]. Since p53 and Rb proteins play an essential role in the regulation of the cell cycle, the inactivation of these proteins by HPV disrupts the normal cell cycle regulation, allowing the proliferation of infected cells and contributing to the development of cervical cancer [10, 11].

TC-1 cells constitute a murine cancer cell line derived from the transfected epithelial cells of lungs of C57BL/6 mice with the HPV-16 *E6*/*E7* oncogene [12]. These cell lines are highly significant due to their ability to replicate key characteristics of HPV-associated diseases, making them an essential model system for studying HPV-related malignancies [13].

Current standards of treatment for cervical cancer comprise of procedures such as surgery, radiation, and chemotherapy [14]. Traditional chemotherapy methods prove ineffective in producing desired therapeutic effects and often result in significant systemic toxicity [15]. Consequently, exploring a treatment approach derived from natural products presents itself as a promising avenue [16]. Curcumin, a widely recognized natural compound, demonstrates remarkable anti-cancer properties through the modulation of various genes and proteins associated with proliferation, oncogenesis, and resistance to chemotherapy [17, 18]. Despite its therapeutic potential, curcumin's poor aqueous solubility, rapid degradation, and inadequate bioavailability have hindered its clinical application [19, 20]. To address these challenges, researchers have turned to nanotechnology, and specifically, curcumin nanoemulsions have emerged as a promising delivery system.

Nanoemulsions are colloidal dispersions of oil in water or water in oil with droplet sizes typically ranging from 20 to 200 nm. They offer several advantages, including improved stability, enhanced solubility of lipophilic compounds, and increased bioavailability [21]. In the context of curcumin, nanoemulsions have shown great potential in overcoming its limitations and maximizing its therapeutic benefits [22, 23]. To address and overcome curcumin's limitations, we developed a curcumin nanoformulation based on an oil-in-water nanoemulsion [24].

Given the significance of cervical cancer, the present investigation assessed the impact of curcumin nanoemulsion on cell viability, the stimulation of apoptosis, and the modulation of *E6*, *E7*, *Rb*, and *p53* at the RNA levels within HPV + TC-1 cancer cells in vitro. Additionally, the anti-tumor activity of this curcumin nanoformulation was evaluated in C57BL/6 female mouse models of cervical cancer bearing HPV + TC-1 tumors.

Methods

Cur-NE preparation

Cur-NE was formulated using the optimized protocol detailed previously [24]. In summary, the preparation process involved forming an oil phase consisting of castor oil, tocopheryl acetate, Tween 80, and polyethylene glycol 400. Curcumin was subsequently incorporated into the oil phase, which was then stirred at 50 °C to ensure homogeneity. Water was gradually added to the mixture and further processed with a probe sonicator for 15 min to achieve the desired nanoemulsion. The characterization of the nanoemulsions was performed by dynamic light scattering (DLS), transmission electron microscopy, and High-performance liquid chromatography (HPLC).

Cell culture

The TC-1 cell line, established from initial lung epithelial cells of C57BL/6 mice, and the MC3 T3 cell line, an osteoblastic lineage derived from the calvaria of a C57BL/6 mouse, were obtained from the Pasteur Institute of Iran. Both cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) enriched with 10% fetal bovine serum (FBS, Gibco) and incubated at 37 °C in a 5% CO2 atmosphere.

Cytotoxicity assay

The cytotoxic effects of Cur-NE and curcumin on TC-1 cancer cells and MC3 T3 normal cells were assessed using the MTT assay, following the methodology outlined in a previous study [24]. In brief, after seeding 15×10^3 cells per well in a 96-well plate, treatments with Cur-NE and Cur were applied in triplicates for 48 h under controlled conditions. Formazan formation was

quantified spectrophotometrically, with cytotoxicity calculated using OD measurements at 540 nm.

Cellular apoptosis analysis

Flow cytometry was employed to assess cellular apoptosis through annexin V/propidium iodide (PI) staining. TC-1 and MC3 T3 cell lines were treated with Cur and Cur-NE at 55 µg/ml and 35 µg/ml, respectively, for 48 h, both based on IC50 (mean inhibitory concentration) values determined in a prior study [24]. After treatment, the staining of cells was carried out by the Annexin V/PI apoptosis detection kit in accordance with the guidelines established by the manufacturer (eBioscience, Thermo Fisher Scientific). Apoptotic and necrotic cell populations were subsequently analyzed using a flow cytometer (FACS Calibur, USA). The resulting data analysis was done utilyzing FlowJo software (version 10, Treestar, USA).

RNA extraction and cDNA synthesis

Total RNA extraction from both treated and untreated TC-1 and MC3 T3 cells exposed to curcumin and curcumin nanoemulsion (Cur-NE) (10–80 µg/mL) was conducted using ROJE Technologies kit. Subsequently, single-stranded complementary DNA (cDNA) was synthesized through a cDNA reverse transcription kit (ParsToos), following the manufacturer's specified protocol, the specific primers for all genes were demonstrated in Table 1.

In vivo tumor model and treat protocol

To study the anti-tumor activity of the curcumin nanoemulsion, 24 female C57BL/6 mice (5–6 weeks old, ~20 g) were used to develop tumor models. 2–3 $\times 10^6$ TC-1 cells per mouse were administered subcutaneously into the right flank of each mouse. When tumors became detectable on day 6, the mice were categorized into four groups (six mice in each group)and treated intravenously with 50 mg/kg of Cur-NE, Cur, blank nanoemulsion, and PBS daily for three weeks, respectively [25]. The value of injection volume was calculated using this formula:

Injection volume = Dosage (mg/kg) x Mouse weight (kg)/treatment concentration (mg/mL).

The volume of tumors was measured every three days during the treatment period. Mean survival time (MST) refers to the time at which half of the mice have died. The tumor volume and increased life span percentage (ILS%) of mice were determined by the following formula [26]:

$$\text{Tumorvolume} = \left(\text{length} \times \text{width}^2\right)/2$$

$$\text{ILS\%} = [(\text{MST of treated mice}/\text{MST of control mice}) - 1] \times 100$$

Results

Characterization of Cur-NE

The results revealed the stability and uniform dispersion of the particles with an average particle size of 52.5 nm, a polydisperse index (PDI) of 0.03, a zeta potential of −13.1 mV, and a drug content of 96.3% [24].

Apoptosis assay

To examine the apoptosis effects of Cur-NE and Cur on TC-1 and MC3 T3 cells, Flow Cytometry analysis was carried out. According to the data in Fig. 1, Cur-NE could induce 46.3% apoptosis in cancerous TC-1 cells at an IC50 of 35µg/ml. In comparison, Cur could induce

Table 1 Characterization of the primer sequences used in this study for qPCR

Gene	Forward primer	Reverse primer	Product length (bp)
E6	AGC GAC CCA GAA AGT TACC	AAG CAA AGT CAT ATA CCT CACG	120
E7	CAG AGG AGG AGG ATG AAA TAG ATG	CGT GTG TGC TTT GTA CGC	126
p53	ACA TGA CGG AGG TCG TGA GA	TTT CCT TCC ACC CGG ATA AG	97
Rb	ACT CCG TTT TCA TGC AGA GAC TAA	GAG GAA TGT GAG GTA TTG GTG ACA	90
GAPDH	TTC AAC GGC ACA GTC AAGG	GTA GAC TCC ACG ACATAC TCAGC	131

(See figure on next page.)

Fig. 1 **A** Flow cytometry results of TC-1 and MC3 T3 cells treated with 35 µg/ml curcumin nanoemulsion and 55 µg/ml curcumin. The regions included Q1, Q2, Q3 and Q4 consisting cell necrosis, late apoptosis, early apoptosis and live cells, respectively. **B** 46.3% of TC-1 cells treated with 35 µg/ml curcumn nanoemulsion became apoptotic. Free curcumin at a higher concentration (55 µg/ml) could induce apoptosis in 36.65% of TC-1 cells. The apoptosis test for MC3 T3 cells exhibited that curcumn nanoemulsion (35 µg/ml) and curcumin (55 µg/ml) had no significant effect on the cell apoptosis (Significant at * < 0.05, *** $P < 0.001$, **** $P < 0.0001$, and ns = not significant)

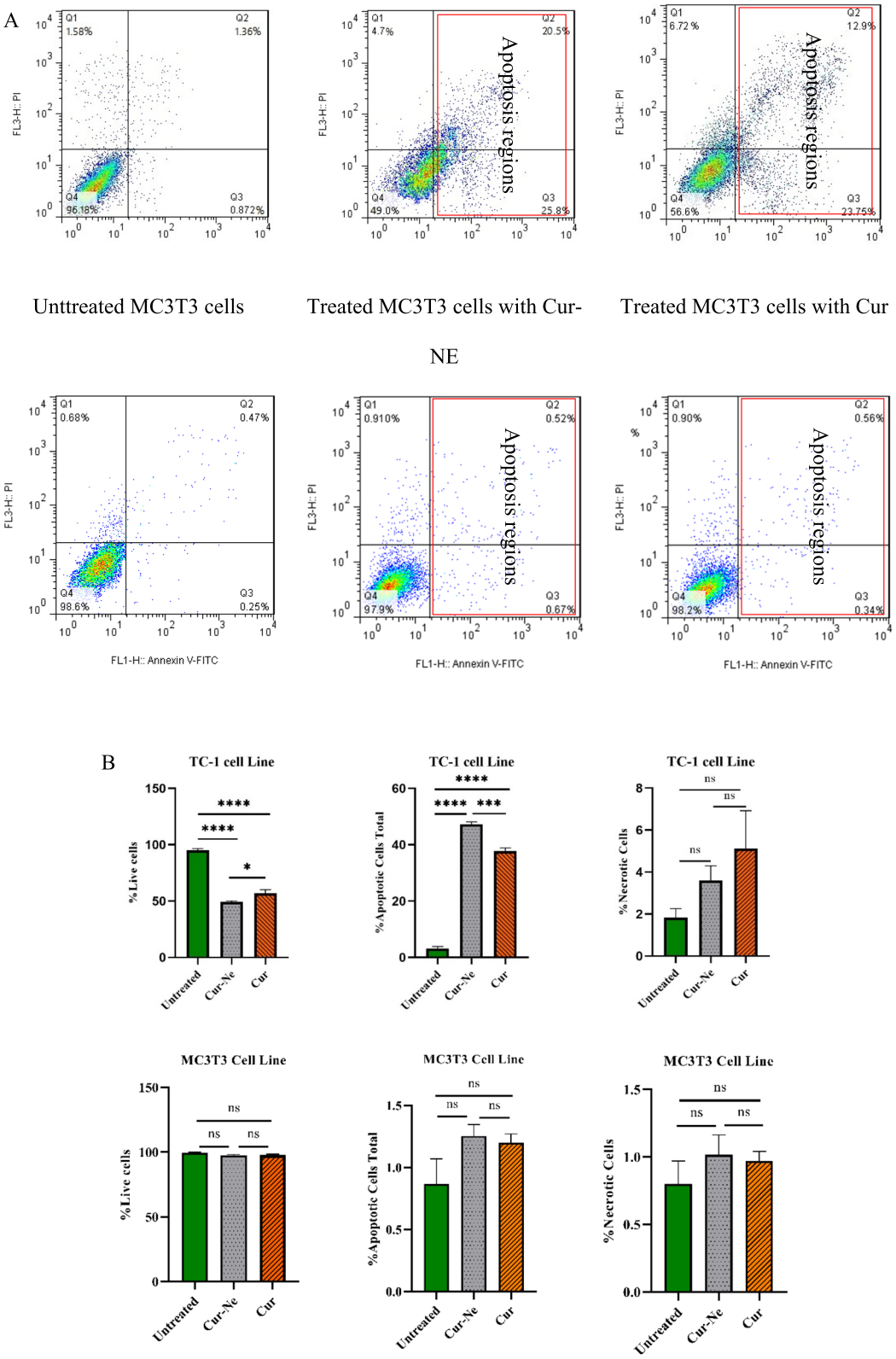

Fig. 1 (See legend on previous page.)

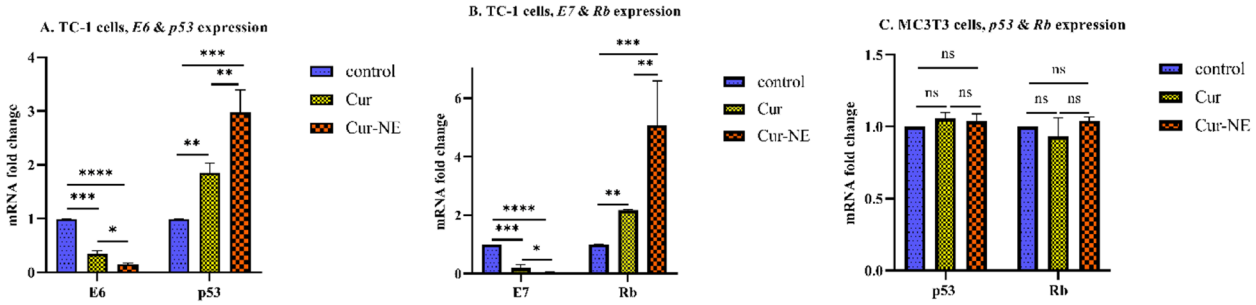

Fig. 2 **A** Gene expression of *E6*, *E7*, *p53* and *Rb* and (**B**) TC-1 cells; (**C**) Gene expression of *p53* and *Rb* in MC3 T3 (Significant at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, and ns = not significant)

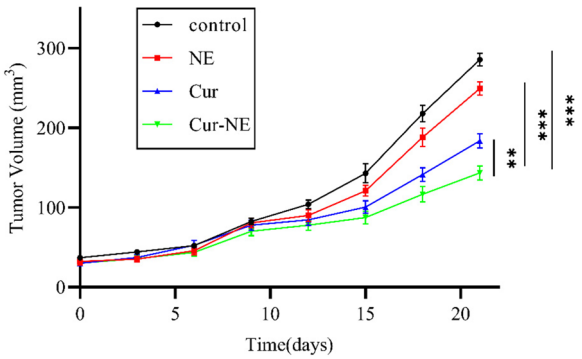

Fig. 3 Tumor development curve of tumor bearing mice in each treatment group (Significant at **$P < 0.01$, *** $P < 0.001$)

apoptosis in 36.65% of TC-1 cells at a higher concentration (55 µg/ml). Therefore, the cytotoxicity of Cur was significantly increased in Cur-NE and a significant difference was observed between Cur-NE and Cur ($P < 0.001$). On the other side of this assessment, no considerable apoptosis effect was observed in normal cells through treatment by both Cur and Cue-NE and there was no significant difference statistically between them.

E6, *E7*, *p53* and *Rb* expression assessment

The effect of Cur-NE and Cur on TC-1 gene expression was evaluated by measuring *E6*, *E7*, *p53* and *Rb* expression under treatment of Cur-NE and Cur during 48 h. On one hand, the results in Fig. 2 demonstrated that Cur-NE downregulated the expression of *E6* and *E7* oncogenes while this reduction is meaningfully different from Cur ($P < 0.05$); on the other hand, it upregulated the expression of *p53* and *Rb* significantly in comparison with Cur ($p < 0.01$).

In vivo anti-tumor efficiency assessment

As shown in Fig. 3, Cur-NE significantly reduced tumor growth in the mice group in comparison with the control mice group ($p < 0.001$) and the Cur mice group ($p < 0.01$). The MST of mice in the PBS, blank nanoemulsion, Cur, and Cur-NE groups was 28.6, 32, 38.3, and 47.3 days, respectively (Table 2). Therefore, the ILS% for the Cur and Cur-NE mice groups, compared to the PBS group, was 37.2% and 65%, respectively. The ILS% of the Cur-NE mice group was significantly higher than the Cur mice group ($p = 0.004$).

Table 2 The therapeutic effectiveness of curcumin nanoemulsion, curcumin, free nanoemulsion and PBS on the MST and ILS % in mice with HPV + TC-1 tumors (Significant at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and ns = not significant)

Parameters	Formulation Type						
	Cur-NE			Cur		NE	PBS
MST	47.3			38.3		32	28.6
P value	Cur-NE vs Cur	Cur-NE vs NE	Cur-NE vs PBS	Cur vs NE	Cur vs PBS	NE vs PBS	
	< 0.01	< 0.01	< 0.001	< 0.05	< 0.01	ns	
ILS%	65			37.2		11.6	-
***P* value**	Cur-NE vs Cur					Cur-NE vs NE	
	< 0.01					< 0.0001	

Discussion

In the current study, the significance of cervical cancer in HPV-associated malignancies led to an exploration of the effect of Cur-NE on HPV + TC-1 cells in vitro and in vivo. According to our previous study, it is figured out from the results of MTT assay that the blank nanoemulsion has no cytotoxicity effect on normal MC3 T3 cells. This result could guarantee the safety of materials of this formulation of nanoemulsion. Moreover, from these assessments it was understood free Cur and also Cur-NE have no significant cytotoxicity on normal cells that could be accounted as a considerable characterization of these materials in comparison to chemotherapy treatment that has cytotoxicity effects on normal cells [24]. The results of this study demonstrate that Cur-NE, a safe and biocompatible substance, modulates the expression of oncogenes and tumor suppressor proteins involved in the viral cancer cell cycle, potentially inducing apoptosis and inhibiting tumor growth in pre-neoplastic lesions. Given the critical role of *E6* and *E7* expression in early stages of malignancy, where viral genome integration is more prominent, Cur-NE presents a promising therapeutic strategy in these pre-cancerous lesions [27, 28].

Curcumin is recognized for its anti-inflammatory and chemopreventive properties so that various studies and preclinical trials demonstrating its dose-dependent efficacy in both chemoprevention and chemotherapy [29–32]. In 2016, Zaman et al. demonstrated the cytotoxicity of curcumin in cervical cancer cells in a manner dependent on time and concentration [33]. Another study by Pourhanifeh et al. showed the capability of curcumin to alter cellular metabolism and cell cycle in HeLa cells [34]. Research over the past thirty years highlights challenges associated with curcumin, including poor bioavailability, hydrophobicity, inefficient cellular uptake, and rapid metabolism [35, 36]. Numerous experiments, including nano-carriers, have been conducted to address these issues [37, 38]. Yallapu and colleagues revealed that poly lactic-co-glycolic acid nano curcumin could act efficiently as a treatment in ovarian and breast [39]. A practical approach to deliver curcumin is nanoemulsion formulation. In the previous study, we developed a curcumin nanoemulsion (Cur-NE) to deliver curcumin and overcome its limitations. In general, our data in this study confirmed not only the dose-dependent behavior of Cur but also that of Cur-NE, as reported in our previous study, showing that cell viability decreased as the concentrations of Cur and Cur-NE increased [24].

When evaluating the impact of Cur and Cur-NE on the viability and apoptosis of TC-1 cells, Cur-NE was significantly more effective than Cur. Cur-NE facilitated the progression of the apoptosis process in TC-1 tumor cells while demonstrating no noteworthy effect on non-cancerous MC3 T3 cells. This observation aligns with findings from previous studies involving other types of cancer cells [40, 41]. According to the results of the work by Seyed Hosseini and colleagues, nanocurcumin was more effective than curcumin in inducing ovarian cancer cell death, while there was no significant change in normal cells at the same concentration [42]. The improved efficacy of Cur-NE compared to curcumin can be attributed to the increased cellular uptake due to the nano-sized particles and the improved biostability due to the encapsulation of curcumin [43].

Moreover, individual treatments of TC-1 and MC3 T3 cells with blank nanoemulsions revealed little cytotoxicity and no substantial impact on cellular viability. Consequently, it can be inferred that Cur-NE, at the investigated concentration, is safe for normal cells in vitro. Several factors contribute to curcumin's selective toxicity toward cancer cells while sparing normal cells. First, the reduction of intracellular glutathione in cancer cells through buthionine sulfoximine leads to elevated levels of reactive oxygen species (ROS), which sensitizes cancer cells to curcumin [44]. Additionally, curcumin targets molecules that are more abundantly expressed in cancer cells, further enhancing its selective anticancer effects [45].

TC-1 cells originate from C57BL/6 mouse primary lung cells immortalized with HPV-16 *E6* and *E7* oncogenes and are commonly employed in numerous research studies for assessing various treatment approaches for cervical cancer [44]. The pathogenesis of HPV is prominently influenced by the pivotal roles played by *E6* and *E7* [45]. These genes encode proteins that interact with the tumor suppressors p53 and Rb, resulting in their suppression [46]. This process disrupts cell cycle regulation, leading to irregular cell proliferation and ultimately to cancer [47].

The expression of the viral oncogenes *E6* and *E7* is crucial for the initiation and progression of cervical cancer [48, 49].In the present investigation, treatment of TC-1 cells with Cur-NE resulted in a significant reduction in *E6* and *E7* expression compared to free curcumin. Since it was reported in this study that Cur-NE significantly induces apoptosis in TC-1 cells compared to Cur and tumor maintenance is influenced by multiple factors, the apoptosis-inducing effect of Cur-NE presents a promising way for cervical cancer treatment.

The extension of the life span observed in mice treated with Cur-NE was notably higher than that in both the free curcumin and control groups (PBS and free nanoemulsion treated groups). This disparity can be ascribed to the extended duration of circulation due to the curcumin nanoemulsion formulation protecting curcumin from degradation, thereby enhancing the bioavailability of the drug to cells.

The superior tumor-suppressive effect of Cur-NE compared to free curcumin can be attributed to nanoemulsion formulation. Indeed, the nanoemulsion formulation may also enhance transmembrane transport, thereby improving cellular uptake and therapeutic efficacy [50].

Consistent findings were reported by Li et al., indicating that a nanoemulsion formulation enhanced the antitumor efficacy of curcumin in the S-180 murine lung neoplasm model [51].

Overall, our data demonstrated the potential of curcumin nanoemulsion to induce apoptosis and suppression of HPV + TC-1 cancer cells and inhibition related tumors growth, suggesting that curcumin nanoemulsion is a potential treatment for cervical cancer and possibly for intraepithelial neoplastic lesions. Finally, additional studies on Histological tests are recommended to investigate the toxicity of curcumin nanoemulsion on the liver and kidney.

Conclusion

In conclusion, this research represents an advance in applying curcumin-based nanoformulation for combating cancer cells. Our results indicate that Cur in an oil/water emulsion inhibits the proliferation of cervical carcinoma cells and modulates the expression of various oncogenic and tumor suppressor proteins implicated in cervical cancer pathogenesis. In vivo experiments also confirm the power of Cur-NE in reducing tumor burden. Thus, the curcumin nanoemulsion represents a promising and innovative chemopreventive and therapeutic approach for treating cervical carcinoma.

Abbreviations

HPV	Human papillomavirus
Cur	Curcumin
Cur-NE	Curcumin nanoemulsion
NE	Nanoemulsion
ILS	Increased life span percentage
MST	Mean survival time

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Authors' contributions

M.K. Doing experiments, Investigation, Writing the original draft, methodology, Conceptualization, data analysis, M.P. Supervision, Review & editing, Validation, Conceptualization, data analysis, Methodology, N.M.K. Review & editing, Methodology M.Q. Review & editing, Methodology M.H.J. Review & editing, Methodology.

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Data availability

Data will be made available on reasonable request from corresponding author.

Declarations

Ethics approval and consent to participate

This study has an ethical approval (code number IR.IAU. PS.REC.1400.042) from Tehran Islamic Azad Medical Sciences University.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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