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PTEN and TRAIL genes loaded zein nanoparticles as potential therapy for hepatocellular carcinoma

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ABSTRACT

Gene therapy is one of the recent approaches in treatment of hepatocellular carcinoma (HCC). Development of a vector or vehicle that can selectively and efficiently deliver the gene to target cells with minimal toxicity is an urgent demand. In the present study, phosphatase and tensin homolog (PTEN) and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) genes were loaded to zein nanoparticles (ZNPs). The formulated PTEN and TRAIL-loaded ZNPs were tested for their \textit{in vitro} and \textit{in vivo} potential antitumor efficacy using liver tumor cells (HepG2) and HCC-induced rats as animal model. Also, mRNA expression of p53, VEGF and MMP-2 were carried out as markers of apoptosis, angiogenesis and metastasis in animal liver tissues. The results of the study showed that both PTEN and TRAIL-loaded ZNPs proved anti-proliferative activity against HepG2 cell lines with IC\textsubscript{50} values of 0.09, 0.25 \textmu g/ml, respectively. \textit{In vivo} assay confirmed decrease in mRNA expression of both VEGF and MMP-2 with increased in P53 expression level in liver tissues of the treated animals. Therefore, authors introduced new integration between gene therapy and nanotechnology in the form of PTEN and TRAIL-loaded ZNPs that proved potential to be used in gene therapy for the treatment of HCC.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of cancer in the world and the second leading cause of cancer-related death [1]. Effective therapy for HCC is currently lacking, creating an urgent need for new treatment strategies. Gene therapy approaches that rely on the transduction of cells with genetic materials, such as apoptotic genes (e.g. P53, PTEN, TRAIL), suicide genes (e.g. herpes simplex virus thymidine kinase HSV-TK), genes coding for antiangiogenic factors or immune-modulatory molecules, small interfering RNA (siRNA) or oncolytic virus, may provide promising strategies [2,3]. Success in gene therapy is largely dependent on the production of a vector that can selectively and efficiently deliver a gene to target cells with minimal toxicity [4].

Nanocarriers, because of their high ratio of surface area to volume ratio, show improved pharmacokinetics and biodistribution of therapeutic agents leading to minimized toxicity owing to their preferential accumulation at the target site [5]. Also, their nanometer size and high permeability lead to the evolution of an ideal gene delivery system; with highly efficient transport mechanism that delivers exogenous genetic material to the tissue-specific site [6]. Ideally, a nanocarrier should be capable of providing extended blood circulation, delivering the active moiety at the targeted site and bypassing the endosome–lyosome processing. They might also increase the stability of a variety of therapeutic agents, such as peptides, oligonucleotides, etc. [7,5].

Protein nanoparticles (NPs) offer a number of advantages including biocompatibility and biodegradability. It was proven that using such biodegradable materials minimizes the possibility of hypersensitivity reactions and provides good tissue compatibility [5]. As well, they can be prepared under mild conditions without the use of toxic chemicals or organic solvents [8].

Zein is a prolamine-rich protein (obtained from maize) that is approved by the FDA for human applications. It contains a high proportion of hydrophobic amino acids, proline and glutamine, as well as, high polar glutamine content [9,10]. The unique structure of zein is closely related to its physicochemical properties and the self-assembly mechanism of zein-based nanosystems. More than 50% of the amino acids comprising zein are hydrophobic, which render it insoluble at physiological conditions and capable of sustained release of the encapsulated compound; on the other hand, zein also has a high polar, protonable side chains. With its amphiphilic character, the hydrophobic regions of zein can cause aggregation into colloidal particles, and the polar side chains allow for interaction with DNA [10,11].

Moreover, Zein and its degraded products have good cell compatibility and can enhance cell proliferation [12,13]. Additionally, it has the advantages over synthetic nanomaterials for its absorbability and the lower toxicity of its degradation products [14]. Furthermore, zein-based nanoparticles (ZNPs) formulation involves several techniques; of which the coacervation technique was selected owing to its simplicity, it does not utilize neither toxic solvents nor high temperatures, and therefore, it aids both final product safety and loaded drug stability [10].

In general, various formulation techniques have been utilized for NPs formulation such as chemical cross-linking,
emulsion–solvent evaporation technique, spray drying and supercritical antisolvent technique [15]. Some advanced techniques were recently investigated such as using single and coaxial electrospray techniques to encapsulate peptides into near mono-dispersed spherical, nanocarriers [16]. Also, gaseous vehicles engineered using electric fields with variable sizes can be used to layer encapsulate proteins due to low shear e.g. Bovine Serum Albumin [17]. Furthermore, several other electro-hydrodynamic atomization technologies are rapidly emerging as promising techniques to formulate complex micro- and nanostructures [18,19].

ZNPs have been formulated with various therapeutic agents such as ivermectin, coumarin, and 5-fluorouracil (5-FU) [20]. Using DNA as therapeutic agent, Regier et al. [10], proved that formulated ZNPs are mostly homogeneous matrix systems in which DNA can be either surface-associated on the zein spheres, but the majority of the DNA remained intact and encapsulated. Zein is expected to enhance DNA hepatocellular internalization through two mechanisms: size-dependant passive targeting and furthermore, the N-terminal region of γ-zein interacts with cell membranes serving as a peptide carrier for DNA across cell membranes [21].

Most of cancer types are known to be due to defects in the apoptotic signaling resulting from mutations or imbalance in the expression of oncogenes and tumor suppressor genes; this usually leads to the resistance of the malignant cells to apoptosis. Consequently, induction of apoptosis by introducing genes encoding an inducer, mediator or executioner of apoptosis is among the most common approach employed in cancer gene therapy [22].

TNF-related apoptosis-inducing ligand (TRAIL) is a cytokine that is produced and secreted by most of the normal cells. It has a unique ability to induce apoptosis in a wide range of transformed cell lines but not in normal tissues. TRAIL is well tolerated when given to healthy animals; on the other hand, multiple injections of soluble TRAIL into mice beginning the day after tumor implantation can significantly suppress the growth of the tumors, but large amounts of soluble TRAIL may be required to inhibit tumor formation [23].

The transfer of TRAIL cDNA (complementary DNA) to cancer cells by using nonreplicating adenoviral vector have been previously reported, [24]; however, little is known about the use of a protein NPs as a safe natural nonviral vector for transferring and targeting TRAIL to cancer cells, which was one of the main goals of the current study.

The PTEN (phosphatase and tensin homolog deleted from chromosome 10) tumor suppressor was identified by homozygous deletion mapping of the human chromosome 10q23 in cancer [25,26]. It is one of the most commonly silenced genes in HCC [27]. It is often down regulated in advanced cancers and metastases [28]. PTEN gene is a tumor suppressor gene that helps to regulate the cycle of cell division and prevent uncontrolled cell growth mainly by antagonizing the action of phosphatidylinositol-3-kinase (PI3K). Therefore, it antagonizes the many signaling pathways that rely on the PI3K activity; the most important signaling cascade in mammalian cells [29]. It is one of the most commonly silenced genes in HCC [25,26]. It is often down regulated in advanced cancers and metastases [28]. PTEN gene is a tumor suppressor gene that helps to regulate the cycle of cell division and prevent uncontrolled cell growth mainly by antagonizing the action of phosphatidylinositol-3-kinase (PI3K). Therefore, it antagonizes the many signaling pathways that rely on the PI3K activity; the most important signaling cascade in mammalian cells [29]. It is one of the most commonly silenced genes in HCC [25,26].

Material and methods

Material

Plasmid preparation

Two different plasmids encoding for PTEN and TRAIL were used, PTEN-GFP plasmid (Addgene plasmid 13039, submitted by Dr. Alonzo Ross, University of Massachussets) and pEGFP-TRAIL plasmid (Addgene plasmid 10953, submitted by Dr. Bingliang Fang, University of Massachussets) were supplied from Addgene (Cambridge, MA) transformed in Escherichia coli strain of DH5α. [31,32]

Reagents and chemicals

Zein (protein from corn, pharmaceutical grade F4400C, approximate molecular weight: 35,000Da) was a kind gift from Flo Chemical Corporation, MA. RT-qPCR assay: RNeasy® Mini-Kit (50) Isolation System (Qiagen, Germany) for RNA extraction. SensiFAST™ cDNA Synthesis Kit (Biotin reagent,Luckenwalde, Germany). KAPA SYBR® FAST qPCR Kits (Kapa Biosystems, Woburn, MA). Primer sequences for PS3, VEGF, MMP-2 and GAPDH (Biosearch technology, Petaluma, CA). AFP ELISA Kit (USCN Life Science, Houston, TX). HPLC grade of CCl₄, absolute Ethanol, HCl and Glacial acetic acid and high molecular analytical grades of Diethyl nitrosamine (DEN), sodium hydroxide, Ethylenediaminetetraacetic acid (EDTA), Glucose, Potassium acetate, Sodium dodecyl sulfate (SDS), and Tris (hydroxymethyl) aminomethane (Tris base) were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture

Human-transformed cell lines from liver (HepG2 cells) were purchased from the Holding Company for Biological Products and Vaccines (VACSERA), Giza, Egypt. Cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 100 μg/ml streptomycin, 100 μg/ml penicillin and 10% (w/v) heat-inactivated fetal bovine serum in a humidified (90–95%), CO₂ atmosphere (5% v/v) at 37°C. Neutral red was purchased from Sigma Chemical Co. (St. Louis, MO).

Animals

Forty adult female wistar albino rats weighing 110–140 g were obtained from the Holding Company for Biological Products and Vaccines (VACSERA, Cairo, Egypt). Animals were housed under12-h light/dark cycles, with free access to water and standard rat chow. The study was approved by scientific research ethics committee at faculty of pharmacy, Helwan University, Cairo, Egypt.

Bacterial culture and plasmid extraction

Both plasmids carrying bacteria PTEN-GFP and pEGFP-TRAIL were inoculated in Luria-Bertani (LB) broth containing 100 mg/ml ampicillin and 100 mg/ml kanamycin, respectively, and incubated at 37°C overnight. Plasmids were extracted from bacterial culture by alkaline lysis method and analyzed using agarose gel electrophoresis. Plasmids concentrations were measured at 260 nm using Nano-Drop spectrophotometer (Quawell® UV-VIS Spectrophotometer, San Jose, CA) [33].

Preparation of DNA-loaded ZNPs

ZNPs were formulated using the coacervation technique [20] with modifications. Zein was first dissolved in 90% ethanol at pH 3 (pH
Table 1. Composition and evaluation of DNA-loaded ZNPs prepared adapting a $2^3 \times 3^1$ full-factorial design.

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>$X_1 =$ Zein concentration (%)</td>
<td>0.1</td>
<td>... 0.2</td>
<td></td>
</tr>
<tr>
<td>$X_2 =$ DNA:Zein ratio</td>
<td>1:60</td>
<td>1:80</td>
<td>1:120</td>
</tr>
<tr>
<td>Transformed values</td>
<td>−1</td>
<td>0</td>
<td>+1</td>
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<tr>
<th>Formulae and their composition at various independent variables levels</th>
<th>Dependent variables</th>
</tr>
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<tbody>
<tr>
<td>Formula</td>
<td>$X_1$</td>
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<td>---------</td>
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<tr>
<td>F1</td>
<td>+1</td>
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</table>

1PS: particle size.
2ZP: zeta potential.
3EE%: percentage encapsulation efficiency of DNA-loaded ZNPs.
4SD: standard deviation from the mean.

adjusted with 0.1 M hydrochloric acid. Then, 0.5 ml of plasmid DNA in Tris EDTA buffer (TE buffer, pH 8) was added to 2 ml of zein alcoholic solution, followed by dropwise addition of 10 ml double-distilled water (DDW) of PH 4 (pH adjusted with 0.1 M hydrochloric acid) while vortexing. DNA-loaded ZNPs were formed via coacervation during the addition of the DDW at PH 4. Additionally, blank ZNPs were formed without DNA (TE buffer substituted for DNA solution). pH of the resultant ZNPs dispersion was gradually increased to 10 through the addition of 0.1 M sodium hydroxide. Such high pH could induce sufficient surface charge on ZNPs to prevent their aggregation during centrifugation and render the formulated ZNPs easily resuspended following centrifugation. ZP values were then precipitated by centrifugation at 10,000 g for 1 h at room temperature. The supernatant was removed and ZNPs were resuspended in 5 ml DDW [10,34].

$2^3 \times 3^1$ full-factorial design was adopted in order to evaluate and optimize the formulation variables affecting DNA-loaded ZNPs as presented in Table 1. In this design, two factors were evaluated as independent variables levels; zein concentration was tested at two levels (0.1% and 0.2%) and DNA:zein ratio was tested at three levels (1:60, 1:80 and 1:120) and experimental trials were performed at all possible combinations with double replication. Particle size (PS) of ZNPs, their zeta potential (ZP) and DNA entrapment efficiency (%EE) were selected as dependent variables (Table 1). The selected levels of the independent variables were chosen based on preliminary experiments and run order was randomized to protect against the effects of time-related variables and to satisfy the statistical requirement of independence of observations. A significant level of 5% was used as the criterion to reject the null hypothesis. Statistical analyses were performed using the Design-Expert® Software (Version 7.0.0, Stat-Ease Inc, Minneapolis, MN). Furthermore, the values of the dependent variables were statistically optimized with the optimization criteria set to smallest PS, highest Z, and highest DNA-loading efficiency of ZNPs and to satisfy the statistical requirement of independence of observations.

Evaluation of DNA-loaded ZNPs

Particle size
For all formulae, Particle size (PS) measurements as well as the polydispersity index (PDI) were carried out on the diluted formula using Malvern Zetasizer Nanoseries (nanoZS; Malvern Inst. Limited, UK). This depends on dynamic light scattering, which measures the Brownian motion and relates it to the PS using Photon Correlation Spectroscopy. A 2 μl aliquot of diluted nanoparticles was dried onto a 400 mesh carbon-coated copper grid and viewed under the TEM at 120 kV, at 40,000X magnification. All measurements were done using appropriately diluted samples (1:50) with DDW at 25°C and angle of 173° and the individual values for two replicates (each replicate with three determinations) were determined and their mean values were reported.

Zeta potential
Zeta potential (ZP) gives an indication about the surface charge of the particles. ZP measurements were carried out using Malvern Zetasizer Nanoseries. This model uses a combination of laser Doppler velocimetry and phase analysis light scattering (PALS) in a patented technique called M3-PALS. All readings were made at 25°C. After centrifugation, a portion of the freshly prepared ZNPs was re-suspended in DDW then injected into zetasizer capillary cell with electrodes at both ends. ZP values were determined for two replicates (each replicate with three determinations) and their mean values were reported.

Transmission electron microscopy
The morphological feature of the DNA-loaded ZNPs observed using transmission electron microscopy (TEM) (JEM-2100, Japan). About 2 μl aliquot of diluted ZNPs suspension was dried onto a 400 mesh carbon-coated copper grid at 40,000 X magnification by CCD camera model AMT, Optronics camera with 1632 × 1632 pixels formatted as side-mount configuration.

Entrapment efficiency (EE)
In order to determine the entrapment efficiency of DNA in ZNPs formed, DNA was extracted from DNA-loaded ZNPs using a standard phenol/chloroform extraction technique by phenol/chloroform/isoamyl alcohol 25:24:1. Then, UV absorbance of the aqueous layer of resuspended pellet was measured at 260 nm versus blank DNA-free ZNPs [35].

$$EE \% = \left( \frac{\text{amount of the entrapped DNA}}{\text{initial amount of the DNA}} \right) \times 100$$

In vitro cytotoxicity assay of PTEN and TRAIL genes loaded ZNPs in liver tumor cell line by neutral red assay method
The cytotoxicity of both PTEN and TRAIL loaded ZNPs was tested against HepG2 cells by the neutral red assay method as previously...
described [36]. Exponentially growing cells were collected using 0.25% Trypsin-EDTA and plated in two 96-well plates at a concentration of 5000 cells/well. After PTEN and TRAIL genes loaded-ZNPs were added to each well (200 μL of serum-free DMEM containing 10 μg/L of each gene); the cells were incubated at 37°C for 6 h in a humidified atmosphere (90–95%) with 5% CO2. Afterwards, the media were replaced with fresh DMEM containing 10% phosphate-buffered saline (FBS) and incubated for an additional 24 h. Then, media were removed and cells were exposed to 200 μL of 0.0075% neutral red solution for 2 h in humidified atmosphere of 5% (v/v) CO2 at 37°C. Ethanol/water/acetic acid (50:49:1) solution was used to dissolve the NR-stained cells and color intensity was measured at 540 nm in a microplate reader, and 50% inhibition concentration (IC50) was calculated using MasterPlex reader 2010 Hitachi (GIRSS) (Global Institute Research Services and Solutions).

In vivo assay
Forty Wistar albino rats used as animal models were acclimatized to laboratory environment for 7 days. They were housed under 12-h light/dark cycles with free access to water and standard rat chow. The study was approved by scientific research ethics committee at faculty of pharmacy, Helwan University, Cairo, Egypt. HCC was inducted in 30 rats by intraperitoneal (IP) injection of initial single dose (200 mg/kg body weight) of diethyl nitrosamine diluted in castor oil followed by weekly subcutaneous (SC) injections of carbon tetrachloride diluted 1:1 in castor oil at a dose of 0.3 ml/rat (twice/week) for 12 weeks [37]. Following HCC induction in rats, they were subdivided into three groups each of 10 rats and the remaining 10 rats were used as normal control as presented in Table 2.

After 2 weeks of treatment, animals were sacrificed by cervical dislocations, blood samples and liver tissues were collected for assessment of the following:

1. Serum alpha fetoprotein (AFP) by ELISA technique
2. Qualitative and quantitative mRNA expression of PS3, VEGF, MMP-2 in liver homogenate by real time PCR
3. Histopathological examination of liver tissues

**Determination of serum AFP**
AFP was determined in serum by enzyme-linked immunosorbent assay technique, which is a solid phase enzyme-linked immuno-sorbant assay (ELISA) based on the sandwich principle. The microtiter wells were coated with a monoclonal antibody directed towards a unique antigenic site on the AFP molecule [39]. Serum samples containing AFP were incubated in the coated wells with enzyme conjugate, which is an anti-AFP antibody conjugated with biotin. After incubation, the unbound conjugate was washed off.

During the second incubation step, streptavidin peroxidase enzyme complex binds to the biotin-anti-AFP antibody. The amount of bound horseradish peroxidase (HRP) complex is proportional to the concentration of AFP in the samples. The enzyme-substrate reaction is terminated by the addition of sulfuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of AFP in the samples is then determined by comparing the optical density (OD) of the samples to the standard curve.

**Quantitative real-time PCR-based analysis of gene expression**
RNA was extracted from liver tissue homogenates according to manufacturer’s instructions using RNeasy® Mini Kit. cDNA was synthesized from the extracted RNA (0.5–2 μg). RT-qPCR assay was carried out for relative quantification of PS3, VEGF and MMP-2 expression using specific primers for each gene and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping gene. Further, data analysis was performed using an Applied Biosystem with software version 3.1 (StepOneTM). Changes in the target mRNA content relative to GAPDH gene were determined based on the cycle threshold (Ct) level according to [40].

\[
\Delta C_t = C_t \text{assessed gene} - C_t \text{reference gene (GAPDH)}
\]

\[
\Delta \Delta C_t = \Delta C_t \text{sample} - \Delta C_t \text{calibrator (healthy) sample}
\]

\[
RQ (\text{Relative Quantification}) = 2^{-\Delta \Delta C_t}
\]

**Histopathological examination**
Resected liver tissue samples were fixed in 10% formalin in phosphate-buffered saline (PBS) and cut into 5-μm thick slices. Each slice was divided into two or more blocks. Sections were examined microscopically after staining with hematoxylin and eosin (H & E).

**Statistical evaluation of the results**
A full-factorial design; adopted to optimize the formulation and process variables affecting ZNPs formulation; was constructed with biotin. After incubation, the unbound conjugate was washed off.

**Results**
**Evaluation of DNA-loaded ZNPs**
The molecular structure of zein, its amphiphilic character, can explain to its self-assembly to from ZNPs. The hydrophobic regions of zein can cause aggregation into colloidal particles while the polar side chains allow for interaction with DNA to form the DNA-loaded ZNPs. PTEN and TRAIL loaded-ZNPs composed of homogeneous matrix systems in which DNA are mostly physically dispersed inside the particle matrix with some conjugated to ZNPs surface [10,15]. Table 1 presents the mean PS, ZP and EE% of the
formulated DNA-loaded ZNPs. The mean PS of the prepared DNA-loaded ZNPs ranged from 132.5 to 238.5 nm.

ZP measurement allows expectation about the storage stability of the dispersion. Generally, absolute ZP values higher than |30| mV indicates excellent physical stability, values around |20| mV are still considered as deflocculated stable system, while those values ranging between −5 mV and +5 mV undergo fast aggregation. ZP results showed that the prepared DNA-loaded ZNPs were negatively charged with the mean ZP ranged from +37.5 to −24.3. Additionally, the mean entrapment efficiency of the formulated ZNPs varied from 11.73 to 41.59%.

The influences of selected variables on PS, ZP and EE% were statistically analyzed via analysis of variance (ANOVA) using Design-Expert® software and revealed both independent variables proved insignificant effects at p values < 0.05 on the DNA-loaded ZNPs. However, the formulae were optimized to the least PS and maximum ZP and EE and it was found that the optimum formula to use is F2 (zein conc. 0.2% W/V and DNA to zein ratio 1:80) of desirability factor 0.855. So this formula was selected for both subsequent in vivo and in vitro studies.

Furthermore, TEM was carried out to obtain more information about the morphology of the prepared DNA-loaded ZNPs. It revealed that the formulated ZNPs have solid dense structure, round and homogenous shape with rough surface that may be due to surface-adsorbed DNA on ZNPs (Figure 1).

**In vitro cytotoxicity assay of both PTEN- and TRAIL-loaded ZNPS**

Treatment of the liver tumor cell lines with either PTEN- or TRAIL-loaded ZNPs lead to decrease in the proliferation rate and cell viability of the treated cells in concentration-dependent manner compared to untreated one after 72-h incubation with remarked increase in the activity of PTEN-loaded ZNPs (IC50 = 0.06 µg/ml) than that of TRAIL (IC50 = 0.25 µg/ml) (Figure 2).

**Serum levels of AFP in different animal groups**

Serum levels of AFP increased significantly in HCC animal group (group 1) compared to the healthy control (group 4) (p < 0.001). On the other hand, the treated animals (groups 2 and 3) showed marked decrease in the serum AFP level compared to untreated animals (group 1) (p < 0.001), although they did not reach to the normal levels of healthy control group (p < 0.05) (Figure 3 and Table 3).

**Expression levels of P53, MMP-2 and VEGF in liver homogenate of the studied animal groups**

P53 showed a significant decrease in its expression level in the HCC-induced animals (group 1) compared to healthy control (group 4) (p values < 0.01). However, treatment with both loaded PTEN and TRAIL genes induced the expression of p53 in the treated animals relative to the untreated (group 1) (p < 0.0001, p < 0.01 respectively).

Regarding VEGF, it was highly expressed in HCC-induced animals (group 1). On the other side, the antiangiogenic effects of the loaded genes used in the study was observed in the treated groups (2 and 3) through the significant decrease in the expression level of VEGF compared to untreated animals, (group 1).

Further, treatment with PTEN and TRAIL genes loaded into ZNPs proved significant improvement of the liver metastasis; this was observed through the decrease in the MMP-2 expression in liver homogenates of the treated animals compared to the untreated ones (Figure 4 and Table 4).

**Histopathological examination**

Histopathological examination of liver tissues of the healthy control group showed normal liver cell plated (Figure 5(A)). While that of the HCC untreated group showed loss of architecture, bounders between cells, merging of cells with bad vasculature, high number of inflammatory cells and appearance focal of malignancy including very common multiple prominent nucleoli, pleomorphic nucleus with hyper chromatism and abnormal mitosis (Figure 5(B)).

On the other hand, the liver tissues from the HCC group treated with either PTEN- or TRAIL-loaded ZNPs revealed almost normal liver cell plates with intervening normal sinusoids and normal portal tracts, associated with observed decrease in both,
number of inflammatory cells and in focus of malignancy of prominent and multiple nucleoli (Figure 5 (C,D)).

**Discussion**

Hepatocellular carcinoma (HCC) is a widely spread health dilemma that is not easily manipulated which represents an urgent need for an effective eradicating therapy. Gene therapy is one of the recent approaches in treatment of HCC. However, the success of gene therapy is largely dependent on the development of a vector or vehicle that can selectively and efficiently deliver the gene to target cells with minimal toxicity and sustained release [10].

In the present study, ZNPs encapsulating DNA of PTEN and TRAIL genes were prepared using a simple coacervation technique. Coacervation, also called phase separation, is a technique that was introduced by the Dutch scientists Bungenberg de Jong and Kruyt in 1929 [41]. It involves macromolecular aggregation (or phase separation); the separation of solutions into colloidal systems with two liquid phases, one phase rich in polymer (the coacervate) and another lacking polymer, which is brought about by the partial desolvation of a previously dissolved polymer. As previously mentioned, zein is completely soluble in hydroalcoholic solution, but it is insoluble in water; therefore, upon decreasing the ethanol concentration of dissolved zein solutions by the addition of aqueous solutions, the necessary desolvation leads to the hydrophobic regions of zein aggregation and the formation of a zein-rich nanosphere phase [10,42].

Since most of the previous studies indicated that the PS and the electrical properties of the formulated NPs can be controlled by variation of the preoperative parameters, zein concentration in the hydroalcoholic solution as well as DNA: zein ratio were expected to affect PS, ZP and the EE of resultant DNA-loaded ZNPs. Preliminary studies showed that reduction of the initial zein concentration in the hydroalcoholic solution from 1% to 0.5% or 0.2% w/v resulted in a significant decrease in the formed ZNPs size. In order to formulate ZNPs that can passively target the liver and cancerous cells, PS should be approximate 100 nm; therefore, the minimum zein concentrations were selected (0.2% and 0.1%). Such results were in agreement with previous studies [10,20]. However, during the study, statistical analysis revealed that in such minute concentrations, varying zein concentration from 0.1% to 0.2% had no significant effect on the formulated ZNPs size ($p > 0.05$),
Although 0.2% W/W zein concentration produced higher yield and EE. Regier et al., [10] proved that the spheres prepared increased in size linearly as the zein: DNA ratio increased. However, ratios lower than 80:1 show instability and tend to aggregate in physiological solution so this ratio was selected. In the present study, 60:1, 80:1 and 120:1 zein: DNA ratios were selected in order to optimize the condition; however, their effect on either PS or EE were statistically insignificant. Further, the results were optimized to attain the minimal PS, maximum ZP and maximum EE using Design-Expert® 9 software. F2 (0.2% zein concentration and 80:1 Zein: DNA ratio) showed desirability of 0.855; therefore, it was selected for the subsequent both in vitro and in vivo studies.

The current study considered unique in loading PETN and TRAIL on ZNPs and studying their potential effects as novel targeting therapy for HCC. Both liver tumor cell line (HepG2 cells), as well as HCC-induced animal model, were used for in vitro and in vivo testing of the potential antitumor effects of the loaded genes.

In the present study, the ability of both loaded genes to inhibit the proliferation of HepG2 cells was confirmed with remarked higher efficiency of PTEN-loaded ZNPs. This result was in accordance with previous studies that revealed the ability of PTEN to inhibit proliferation, migration and invasion of HepG2 cells mainly through its antagonistic effect on PI3K/Akt pathway that promote cell survival and growth [43,44].

On the other hand, some studies reported the potential anti-proliferative effect of TRAIL on HepG2 cells and others revealed the resistance of HepG2 cells to TRAIL due to the presence of certain molecules that inhibit TRAIL-mediated cell death [45]. However, Sun et al. [46] showed that TRAIL gene transfection potentiates HepG2 cell death mediated through mesenchymal stem cell (MSC). Also, Qi et al. [47] reported the inhibitory effect of adeno-associated virus (AAV) carrying TRAIL on the growth in HCC cell lines.

The HCC in the animal model used in the present study was induced using combination of genotoxic DEN and a pro-fibrogenic agent CCL4 [48,49], so that it can mimic the pathophysiological features of HCC in human, as previously mentioned that cirrhosis considered as the leading major risk factor for HCC [50]. The efficacy of PTEN- and TRAIL-loaded ZNPs on HCC-induced animals was examined by measuring expression levels of P53, VEGF and MMP-2 as apoptotic, angiogenic and metastatic genetic markers.

As previously discussed, one of the main approaches for cancer treatment is to restore apoptotic pathway. The p53 pathway plays an important role in silencing carcinogenesis, as the main pathway for apoptosis induction. P53 is an ideal target for cancer treatment as it is able to induce tumor growth suppression through cell cycle arrest and induction of apoptosis [51,52].

In the present research, a downregulation of P53 expression was observed in HCC-induced animal model; this was in agreement with Van Gijssel et al. [53], who reported the role of p53 downregulation in hepatocarcinogenesis in rats received DEN and CCL4. Many evidences suggested that p53 has a great influence on TRAIL-induced apoptosis in cancer cells via multiple mechanisms [54]. Also, combined TRAIL treatment with chemotherapeutics or ionizing radiation usually results in strongly enhanced cytotoxic effects [54,55]. On the other hand, PTEN controls P53 level by decreasing murine double minute 2 (MDM2)-dependant-p53 degradation [56]. These findings were confirmed in the present study through the upregulation of P53 expression resulted from using the gene-loaded ZNPs in HCC animal model.

HCC is a typical hyper-vascular tumor in which angiogenesis is required for both growth and metastasis. VEGF is one of the strongest stimulatory angiogenic factor that is upregulated in most human tumors [57]. In the present study, PTEN-loaded ZNPs were proven to control and downregulate VEGF expression in HCC-treated rats, this coordinates with several studies that emphasize the important negative regulatory role of PTEN in angiogenesis through downregulation of VEGF expression and decreases secretion through its antagonistic effect on PI3K/Akt pathway [58]. Li et al. [59], reported the inhibitory effect of PTEN on the expression of CCL4 and VEGF-A; on the other hand, it was observed that loss of PTEN in cancer lead to an increased VEGF expression due to
upregulation of HIF-1 \cite{60}. Also, as reported by \cite{61} in their study on HCC cells, they reported enhanced expression of VEGF and increased capacity of the tumor cells to form capillary tube structures as a result of PTEN suppression.

Some reports demonstrated controversial effects of TRAIL on endothelial cell angiogenesis; some studies reported TRAIL as a potent angiogenic factor through its activation of Akt and eNOS which are strong angiogenic mediators \cite{62} and also via NADPH oxidase-4 (NOX4) and nitric oxide-dependent mechanisms \cite{63}. TRAIL is also reported to provide prometastatic behavior in cholangiocarcinoma cells \cite{64} xenograft model of pancreatic cancer \cite{65} due to nuclear factor kappa B (NF-κB)-dependent pathways initiated by TRAIL-signaling cascades.

On the contrary, other studies reported the antiangiogenic effects of TRAIL due to increased expression of the antiangiogenic factor CXCL10 and decreased proangiogenic interleukin-8 cytokine \cite{66}. Also TRAIL inhibits VEGF-induced tumor angiogenesis via increasing caspase-8 activation \cite{67}. On the other hand, TRAIL can inhibit human brain endothelial cell angiogenesis \cite{68} and repress VEGF expression \cite{69}. The present study was in agreement with the last theory since TRAIL suppressed the VEGF expression in the treated HCC animals. All these findings provide evidence that TRAIL can be used as antiangiogenic therapy for treating human tumors with acquired drug resistance.

The matrix MMPs play a key role in extracellular matrix degradation and tumor propagation. MMP-2 is a member of the MMP gene family capable of cleaving extracellular matrix components and molecules. It has been involved in vascularization and metastasis causing the focal proteolysis and breakdown of cell–cell junction and increasing the motility of tumor cells \cite{70}. However, fibrosis resulting from in vitro administration of CCL2 is associated with increasing expression of MMP-2 \cite{71}, this finding was supported by the present study through the treatment of the HCC rats by the tested loaded genes where a marked decrease in the expression levels of MMP-2 has been observed and this explained by the ability of both loaded PTEN and TRAIL genes to inhibit metastasis and tumor invasion in the treated HCC rats.

Also Tian et al. \cite{43,44} supported the downregulation effect of PTEN on MMP-2; on the other hand, Siddiqui et al. \cite{72} proved that TRAIL has no significant effect on MMP-2 in contrast to Cantarella et al. \cite{69} which used a combination of TRAIL with green tea polyphenol EGCG in his study on human glioblastoma A172 Cells.

Conclusions

In the present study, the authors introduced a new integration between gene therapy and nanotechnology in the form of PTEN and TRAIL-loaded ZNPs; which offers potential advancement in the treatment strategy of HCC.

PTEN- and TRAIL-loaded ZNPs provide promising gene therapy for HCC; both proved potential in activating apoptosis, decreasing angiogenesis and metastasis in the liver tissues of the HCC-treated animals. Moreover, they inhibited the proliferation of liver tumor cell lines. These findings were confirmed through the improvement of the liver histopathological features in the treated animals.

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Disclosure statement

The authors report no conflict of interest including any financial, personal or other relationships with other people or organizations that could inappropriately influence, or be perceived to influence, the work in this paper.

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