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Oral administration of naturally occurring chitosan-based nanoformulated green tea polyphenol EGCG effectively inhibits prostate cancer cell growth in a xenograft model

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In preclinical animal models, several phytochemicals have shown excellent potential to be used as effective agents in preventing and treating many cancers. However, the limited bioavailability of active agents could be one reason for their restricted usefulness for human consumption. To overcome this limitation, we recently introduced the concept of nanochemoprevention by encapsulating useful bioactive food components for their slow and sustained release. Here, we report the synthesis, characterization and efficacy assessment of a nanotechnology-based oral formulation of chitosan nanoparticles encapsulating epigallocatechin-3-gallate (Chit-nanoEGCG) for the treatment of prostate cancer (PCa) in a preclinical setting. Chit-nanoEGCG with a size of <200 nm diameter and encapsulating EGCG as determined by dynamic light scattering and transmission electron microscope showed slow release of EGCG in simulated gastric juice acidic pH and faster release in simulated intestinal fluid. The antitumor efficacy of Chit-nanoEGCG was assessed in subcutaneously implanted 22Rv1 tumor xenografts in athymic nude mice. Treatment with Chit-nanoEGCG resulted in significant inhibition of tumor growth and secreted prostate-specific antigen levels compared with EGCG and control groups. In tumor tissues of mice treated with Chit-nanoEGCG, compared with groups treated with EGCG and controls, there was significant (i) induction of poly (ADP-ribose) polymerases cleavage, (ii) increase in the protein expression of Bax with concomitant decrease in Bcl-2, (iii) activation of caspases and (iv) reduction in Ki-67 and proliferating cell nuclear antigen. Through this study, we propose a novel preventive and therapeutic modality for PCa using EGCG that addresses issues related to bioavailability.

Introduction

Although extensive efforts have been put to prevent and treat cancer through the use of bioactive food components and other agents, lack of delivery of desired levels, thus limiting their bioavailability, remains an obstacle for effective clinical outcome (1–3). Therefore, it is essential to find new strategies to deliver the bioactive food components to the human population for the prevention and treatment of cancer. For a natural agent to elicit maximum response, newer approaches are required for increasing the bioavailability and reducing the toxicity after continuous use for extended period of time. Nanotechnology can be useful against cancer as many of the related processes occur at nanoscale (4,5). This delivery system could overcome many biological, biophysical and biomedical barriers (4,6).

An oral route is the most accepted way of drug administration in terms of patient compliance and convenience. However, to date oral delivery of many drugs, and of many active ingredients with high therapeutic value in natural products, has proven to provide poor bioavailability when administered orally (7–9). The main obstacle that hinders the clinical availability of naturally derived compounds is the harsh condition of the gastrointestinal tract, mainly due to physical barriers like the intestinal epithelium and degradation by various enzymes and gastric juices (10,11). Epigallocatechin-3-gallate (EGCG), the natural active ingredient of green tea, is the most widely used natural product and has shown excellent potential in several studies to treat and prevent many cancers including prostate cancer (PCa) (12–18).

The association between green tea and black tea consumption with PCa risk was investigated in a meta-analysis. There was a borderline significant association with PCa in Asian populations for highest green tea consumption versus non/lowest and the pooled estimate reached statistically significant level for case–control but not for prospective cohort studies. It was also found that there was no statistically significant association with PCa for the highest versus non/lowest black tea consumption population (19). To study the association between green tea consumption and the risk of cancer incidence and mortality, all prospective, controlled interventional and observational studies with >1.6 million participants were included. Observational studies and the only included randomized controlled trial suggested that consumption of higher quantities of green tea or green tea extract led to decreased risk of PCa in men (20). For the chemoprevention of PCa, a proof-of-principle clinical trial was conducted to assess the safety and efficacy of green tea catechins (GTCs) in high-grade prostate intraepithelial neoplasia volunteers. Three GTC capsules, 200 mg each, were given everyday. Among the 30 GTCs-treated men, one tumor was with an incidence of 3%, whereas after 1 year, nine cancers were found among the 30 placebo-treated men with an incidence of 30%. Subjects treated with GTC showed values lower than placebo-treated ones, but there was no significant change in total prostate-specific antigen (PSA) between the two arms. International Prostate Symptom Score and quality of life scores of GTCs-treated men with coexistent benign prostatic hyperplasia also improved (21). In continuation of this study, another round of prostate mapping was performed in a subset of these patients. The mean follow-up from the end of GTCs dosing was 23.3 months for placebo arm and 19.1 months for GTCs arm with only 9 from the placebo arm and 13 from the GTCs arm undertook this third prostate mapping. During follow-up, there were three further cancer diagnoses, two in the placebo arm and one in the GTCs arm recommending that the inhibition of PCa progression was long term in these subjects after 1 year of GTCs administration (22). Sanna *et al.* (23) developed targeted nanoparticles in order to selectively deliver EGCG to cancer cells. EGCG-loaded nanoparticles consisting of biocompatible polymers, functionalized with small molecules targeting prostate-specific membrane antigen exhibited selective *in vitro* efficiency against prostate-specific membrane antigen-expressing PCa cells.

It is imperative to look for a carrier system that can protect the natural active ingredient from environmental assault and that has

Abbreviations: Chit-nanoEGCG, chitosan nanoparticles encapsulating epigallocatechin-3-gallate; EGCG, epigallocatechin-3-gallate; ELISA, enzyme-linked immunosorbent assay; GTC, green tea catechin; LC/MS, liquid chromatography/mass spectrometry; PARP, poly (ADP-ribose) polymerase; PCa, prostate cancer; PCNA, proliferating cell nuclear antigen; PSA, prostate-specific antigen; TEM, transmission electron microscopy; TPP, pentasodium tripolyphosphate hexahydrate; VEGF, vascular endothelial growth factor.

the capacity to improve the bioavailability. Utilization of ultramodern nanotechnology to incorporate natural active ingredients for the development of aqueous nanoformulations for oral delivery might be useful for improvement in the clinical outcome. It is important to emphasize that the oral bioavailability of the GTC has been shown to be low, resulting in systemic levels in humans 5–50 times less than concentrations shown to exert biological activities in *in vitro* systems (24). In our earlier study while introducing the concept of nanochemoprevention, we reported significant dose advantage of polylactic acid–polyethylene glycol encapsulated EGCG (nanoEGCG) over non-encapsulated EGCG. NanoEGCG had over 10-fold dose advantage for exerting its proapoptotic and antiangiogenic effects (15). However, this custom-made nanoparticle formulation was designed for systemic delivery, and due to its non-mucoadhesive properties, negative surface charge, non-stimuli sensitive behavior and relatively larger size were unsuitable for oral administration (15). To overcome these problems, we have chosen chitosan nanoparticles suitable for oral delivery of EGCG (Chit-nanoEGCG). We synthesized nanoparticles made up of the natural biopolymer chitosan with encapsulated EGCG, which appeared to be stable in the acidic environment of the stomach and prevented release of EGCG in the stomach. Chitosan nanoparticles have been largely used for efficient oral delivery of numerous drugs due to their characteristic mucoadhesive properties (25–28). Chitosan is a natural linear polysaccharide derived by deacetylation of chitin from shrimp/crabs, insects and fungi and is also known for its non-toxic, non-immunogenic properties (29). It is used as a pharmaceutical excipient, a weight loss supplement, an experimental mucosal adjuvant and a Food and Drug Administration-approved hemostatic dressing (30–33). It has a positive charge due to the presence of NH_2 groups that also provide the necessary properties to adhere to the gastrointestinal tract for a longer time. Thus, chitosan nanoparticles' increased retention time, ultrafine size and ability to release the drug for a longer time make them ideal oral delivery vehicle.

In this study, we report the synthesis and characterization of chitosan nanoparticles with a size in the range of 150–200 nm diameter encapsulating EGCG. These nanoparticles showed a slow release of EGCG in acidic pH (simulated gastric juice) and faster release in simulated intestinal fluid (neutral pH). The antitumor efficacy of this uniquely formulated Chit-nanoEGCG was assessed in subcutaneously implanted tumor xenografts in athymic nude mice. Significant improvement of therapeutic benefit against PCa tumors was observed with this Chit-nanoEGCG compared with its free counterpart.

Material and methods

Materials

Anti-Bax, Bcl-2, active caspases-3, 8 and 9 and poly (ADP-ribose) polymerase (PARP) antibodies were obtained from Cell Signaling Technology (Beverly, MA). Vascular endothelial growth factor (VEGF) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Ki-67 and CD31 antibodies were purchased from Abcam (Cambridge, MA). Human PSA ELISA kit was procured from Anogen (Mississauga, Ontario). Anti-mouse and anti-rabbit secondary antibody horseradish peroxidase conjugates were obtained from Amersham Life Science (Arlington Height, IL). BCA Protein assay kit was obtained from Pierce (Rockford, IL). Novex precast Tris-glycine gels were from Bio-Rad (Hercules, CA). EGCG, pentasodium tripolyphosphate hexahydrate (TPP), heptafluorobutyric acid, formaldehyde, acetonitrile and cellulose dialysis tubing were purchased from Sigma–Aldrich Co. (St Louis, MO). Water-soluble chitosan was obtained from Polysciences (Warrington, PA).

Synthesis of chitosan nanoparticles encapsulating EGCG

To synthesize chitosan nanoparticles, 45 ml of a solution of EGCG (10 mg/ml in deionized water) was added to 5 ml of 1% water-soluble chitosan and stirred for 1 h. Next, 1 ml of a TPP (10 mg/ml in deionized water) was added drop by drop, with constant stirring. The entire solution was then sonicated for ~30 s using a probe sonicator and allowed to stir for ~4 h. This solution containing EGCG nanoparticles was dialyzed to remove the impurities and the free EGCG using a 100 kDa cutoff dialysis membrane. The solution was then lyophilized to get the nanoformulation in powdered form and redispersed in deionized water for further use.

Size measurement by dynamic light scattering

Size distribution of Chit-nanoEGCG in aqueous dispersion was determined by using a Malvern zeta sizer (Malvern Instrumentation Co., Westborough, MA) and was compared with the Chit-nano without incorporation of EGCG (void nanoparticles). Two milliliters of Chit-nanoEGCG/void nanoparticles were placed in a four-sided, clear, plastic cuvette and analyzed directly at 25°C. The size of the nanoparticles was found to be <200 nm in diameter.

Size measurement by transmission electron microscope

The size and morphology of Chit-nanoEGCG nanoparticles were examined by transmission electron microscopy (TEM) using a JEOL JEM-100CX TEM (JEOL USA, Peabody, MA). One drop of the Chit-nanoEGCG solution was mounted on a thin film of amorphous carbon deposited on a copper grid (300 mesh). The solution was air dried, and the sample was examined directly under the microscope.

Determination of entrapment efficiency (loading efficiency)

The amount of EGCG was determined by disintegrating the nanoparticles and measuring the EGCG by liquid chromatography/mass spectrometry (LC/MS). This also helps in determination of the entrapment efficiency of EGCG in the nanoformulation. The redispersed nanoparticles were disintegrated by adding an acetic acid solution (by incubating it in 1% v/v solution for 30 min). The entire solution was passed through a filter Millipore centrifugal device of 100 kDa cutoff with the help of centrifugation, at around 6500 r.p.m. for 15 min to separate the EGCG. The concentration of the centrifugate containing EGCG was determined using LC/MS. The entrapment efficiency was determined by the following formula:

$$\text{Entrapment efficiency (loading)} = \frac{[\text{EGCG}]_f}{[\text{EGCG}]_t} \times 100 \quad (1)$$

where $[\text{EGCG}]_f$ is the concentration of EGCG in the centrifugate and $[\text{EGCG}]_t$ is the theoretical concentration of EGCG (meaning total amount of EGCG added initially).

Release kinetics studies

The cumulative release of EGCG from Chit-nanoEGCG nanoparticles was studied in simulated gastric juice and simulated intestinal fluid. Simulated gastric juice (purchased from Ricca Chemical Company, Arlington, TX) composed of a mixture of sodium chloride and hydrochloric acid with pH around 1.5. Simulated intestinal fluid (purchased from Ricca Chemical Company) contained potassium phosphate monobasic, sodium hydroxide and pancreatin with pH around 6.8. For this cumulative release kinetic study, a known amount of nanoparticles encapsulated EGCG was suspended in 15 ml of simulated gastric juice/simulated intestinal juice. The solution was kept in an incubator at room temperature (25°C). At predetermined time intervals, 500 µl of the solution was filtered through Millipore Centricon tubes containing a 100 kDa membrane to separate released EGCG from the nanoparticles. The amount of released EGCG present in the filtrate was determined by LC/MS.

Protein extraction and immunoblotting

The tumor tissue samples were homogenized in ice-cold lysis buffer [50 mM Tris–HCl, 150 mM NaCl, 1 mM ethyleneglycol-bis(aminoethylether)-tetraacetic acid, 1 mM ethylenediaminetetraacetic acid, 20 mM NaF, 100 mM Na_3VO_4, 0.5% NP-40, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, pH 7.4] with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem, La Jolla, CA). The homogenate was then centrifuged at 14 000*g* for 25 min at 4°C, and the supernatant (whole cell lysate) was collected, aliquoted and stored at −80°C.

For immunoblotting, 30–50 µg protein was resolved over 8–12% polyacrylamide gels and transferred to a nitrocellulose membrane. The blot was blocked in blocking buffer (5% non-fat dry milk/1% Tween 20; in 20 mM Tris-buffered saline, pH 7.6) for 1 h at room temperature, incubated with appropriate monoclonal or polyclonal primary antibody in blocking buffer for 1.5 h to overnight at 4°C, followed by incubation with anti-mouse or anti-rabbit secondary antibody horseradish peroxidase conjugate obtained from Amersham Life Science and detected by chemiluminescence and autoradiography using Bio-Rad Gel-Doc (Bio-Rad Laboratories). Densitometric measurements of the bands in immunoblotting were performed using digitalized scientific software program Image J (National Institutes of Health).

In vivo *tumor xenograft in athymic nude mice*

Ethics statement. The studies were conducted according to the Institutional guidelines for the care and use of animals and were approved by Animal Care

and Use Committee, School of Medicine and Public Health, University of Wisconsin-Madison.

The effects of oral Chit-nanoEGCG were studied *in vivo* using athymic nude mice xenograft. Athymic (*nu/nu*) male nude mice (NxGen Biosciences) were housed under pathogen-free conditions with a 12 h light/12 h dark schedule and fed with an autoclaved diet *ad libitum*. Forty-eight male athymic nude mice (4 weeks of age) were housed four per cage and fed *ad libitum* with autoclaved semi-purified, AIN-76 B-40 diet. A total of 1 million 22Rv1 cells (in 50 µl Dulbecco's modified Eagle's medium + 50 µl Matrigel) were implanted by a subcutaneous injection on left and right sides, below the shoulders (two tumors per mouse). AR-positive 22Rv1 cells were implanted subcutaneously in mice based on the fact that these cells form rapid and reproducible tumors in nude mice and also secrete significant amounts of PSA in the bloodstream of the host (34,35). Mice implanted with cells were randomly distributed into four groups of 12 each. One day post cell inoculation, Group I received void chitosan nanoparticles by oral intubation, five times a week and served as the control. Group II received EGCG (40 mg/kg body wt) by oral intubation, five times a week. Group III received Chit-nanoEGCG (3 mg/kg body wt) and Group IV received Chit-nanoEGCG (6 mg/kg body wt) by oral intubation, five times a week. Treatments were continued until tumors reached a targeted volume of 1200 mm^3. The tumor size was measured by determining two perpendicular dimensions with calipers, and the volume was calculated using the formula $(a \times b^2)/2$, where a is the longer and b is the smaller dimension. The animals were also evaluated for body weight, consumption of food and apparent signs of toxicity. At weekly intervals, phlebotomy was performed to obtain sera for PSA estimation by enzyme-linked immunosorbent assay (ELISA).

PSA estimation by ELISA

The human PSA ELISA kit from Anogen was used for the quantitative determination of PSA levels in serum according to manufacturer's instructions.

Immunohistochemical analysis

Sections (5 µm thick) were cut from paraffin embedded tumor tissues. Immunostaining was performed using specific antibodies with appropriate dilutions and was replaced with either normal host serum or block for negative controls, followed by staining with appropriate horseradish peroxidase-conjugated secondary antibodies. The slides were developed in diaminobenzidine and counter stained with a weak solution of hematoxylin stain. The stained slides were dehydrated and mounted in permount and visualized on Nikon Eclipse Ti system (Nikon Instruments). Images were captured with an attached camera linked to a computer.

Statistical analysis

Results were analyzed using a two-tailed Student's *t*-test to assess statistical significance and *P*-values <0.05 were considered significant. The *P*-values in Figure 3B were calculated by *t*-test followed by analysis of variance and *P*-values <0.05 were considered significant. The percent decrease in serum PSA in Figure 3D was calculated by considering serum PSA value of control group as 100% and then comparing the values of EGCG and Chit-nanoEGCG-treated groups with the control group.

Results

Synthesis and characterization of Chit-nanoEGCG

A representative diagram comparing the size and size distribution of void chitosan nanoparticles and Chit-nanoEGCG by dynamic light scattering is shown in Figure 1. It was observed that the size distribution of both void and Chit-nanoEGCG nanoparticles was ~150 to <200 nm in diameter. The TEM image of control chitosan nanoparticle is shown in Figure 2A. The size and morphology were further confirmed by TEM as shown in Figure 2B, which also supported the fact that the nanoparticles had a size of <200 nm in diameter as evidenced by dynamic light scattering in Figure 1. Additionally, from the TEM picture, it was clear that the nanoparticles were spherical in shape (Figure 2B). We evaluated the size and morphology of void chitosan nanoparticles and found that there was no notable change in size and morphology of chitosan void (control) nanoparticles compared with the Chit-nanoEGCG.

We also developed a method to determine the amount of EGCG encapsulated in the nanoparticles by LC/MS. A graphical representation comparing the release profiles in simulated gastric juice and

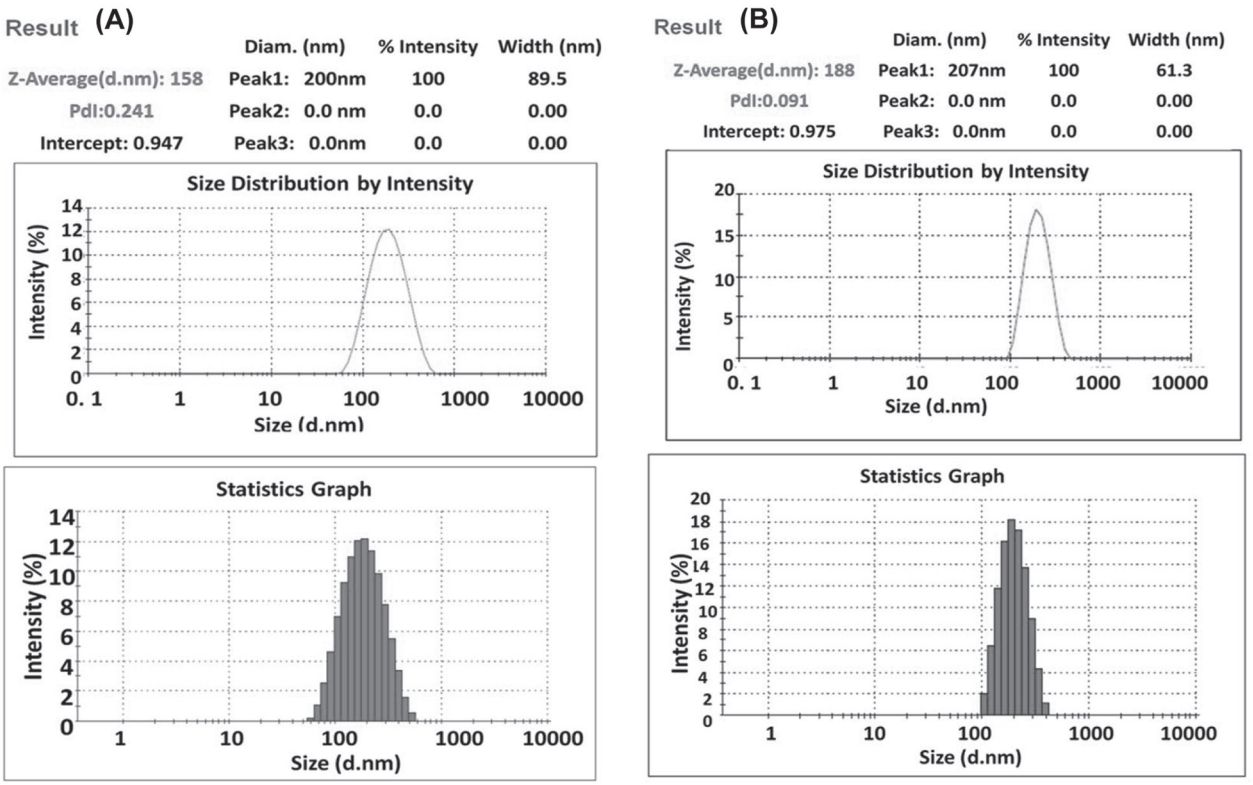

Fig. 1. Size and size distribution measurement by dynamic light scattering of (**A**) void chitosan nanoparticles and (**B**) Chit-nanoEGCG. The upper panel shows the size distribution of the nanoparticles by intensity and lower panel is the histogram of the size distribution from the intensity graph (upper panel). The graph (lower panel) is configured to display a size distribution by intensity graph as a histogram, with logarithmic *x*-axis and linear *y*-axis settings. This graph represents the size statistics of nanoparticles by intensity.

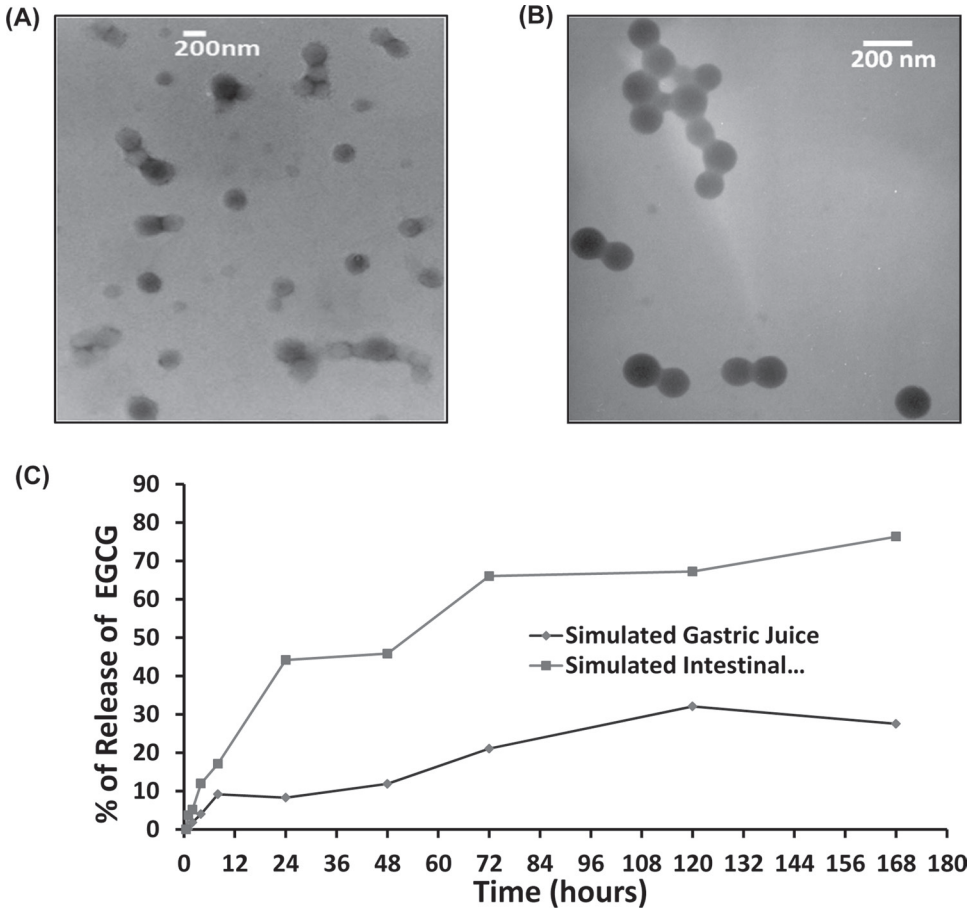

Fig. 2. (**A**) TEM images showing the spherical size and morphology of Chit-nanoEGCG nanoparticles, and (**B**) void chitosan nanoparticles. (**C**) Cumulative release kinetics of EGCG from Chit-nanoEGCG in simulated gastric juice and simulated intestinal fluid.

simulated intestinal fluid is shown in Figure 2C. In the case of oral delivery of an active biomaterial/natural product, the degradation starts in the stomach, due to the acidic pH. One of the most important factors to be kept in mind for carrier-mediated drug delivery through an oral route is that the carrier system must not degrade/release at acidic pH, releasing the entire drug. At the same time, it has to retain its capability to release the drug at neutral pH. To test the stability and release kinetics of Chit-nanoEGCG nanoparticles, we studied the release kinetics in simulated gastric juice and simulated intestinal fluid. It is clear from Figure 2C that the release of EGCG from these nanoparticles is very slow in acidic medium; even at 24 h, only ~10% of EGCG was released. On the other hand, release of intestinal fluid is much faster, and ~50% of EGCG was released in 24 h. Then there was a steady release of EGCG. This is strong evidence that these nanoparticles are capable of avoiding degradation in the stomach, they do not release EGCG and they are capable of releasing EGCG in a neutral medium.

Inhibition of the growth of human prostate carcinoma 22Rv1 cells in athymic nude mice by Chit-nanoEGCG

The treatment of athymic nude mice with nanoEGCG resulted in inhibition of AR-positive 22Rv1 tumor xenograft growth. The appearance of small solid tumors was observed in animals of control group 11 days after cell inoculation. This latency period was prolonged to 18 days in animals receiving EGCG (40 mg/kg body wt) and 25 days in animals receiving Chit-nanoEGCG (3 and 6 mg/kg body wt). This shows that Chit-nanoEGCG has the ability to delay the tumors more effectively than EGCG and shows promise as an agent to enhance the latency period against prostate cancer. There was significant reduction in growth of prostate tumors in EGCG- and Chit-nanoEGCG-treated animals compared with control group (Figure 3A–C). As depicted in Figure 3B, tumor growth, as inferred by computed tumor volume, was significantly inhibited in mice receiving EGCG and Chit-nanoEGCG. In control group, the average tumor volume of 1200 mm^3 was reached in 32 days after tumor cell inoculation. At this time point, the average tumor volume was 514, 310 and 216 in EGCG, Chit-nanoEGCG (3 mg/kg body wt) and Chit-nanoEGCG (6 mg/kg body wt) treated groups, respectively. This showed that there was marked difference in the growth of tumors when animals were treated with EGCG and Chit-nanoEGCG. The inhibition of tumor growth was more pronounced in groups treated with Chit-nanoEGCG than with EGCG alone. The average tumor volume of 1200 mm^3 was achieved 46, 53 and 60 days after tumor cell inoculation in EGCG, Chit-nanoEGCG (3 mg/kg body wt) and Chit-nanoEGCG (6 mg/kg body wt) treated groups, respectively (Figure 3B and C). There was dose-dependent inhibition of tumor growth by Chit-nanoEGCG and it was found to be more effective than EGCG.

Inhibition of PSA secretion in athymic nude mice by Chit-nanoEGCG

During the course of tumor growth in animals at day 2, 8, 14, 20 and 26 after inoculation, blood was collected through the mandibular bleed. Quantitative sandwich ELISA was used to determine circulating PSA levels in mouse serum secreted by 22Rv1 tumor xenografts. PSA is a serine protease with highly prostate-specific expression and is the most widely employed marker in the detection of early PCa. For these reasons, it is considered that agents that could reduce PSA levels may have important clinical implications for PCa. It is currently the most accepted marker for assessment of PCa progression in humans and is being detected in the serum of patients with prostate diseases including prostatitis, benign prostatic hypertrophy

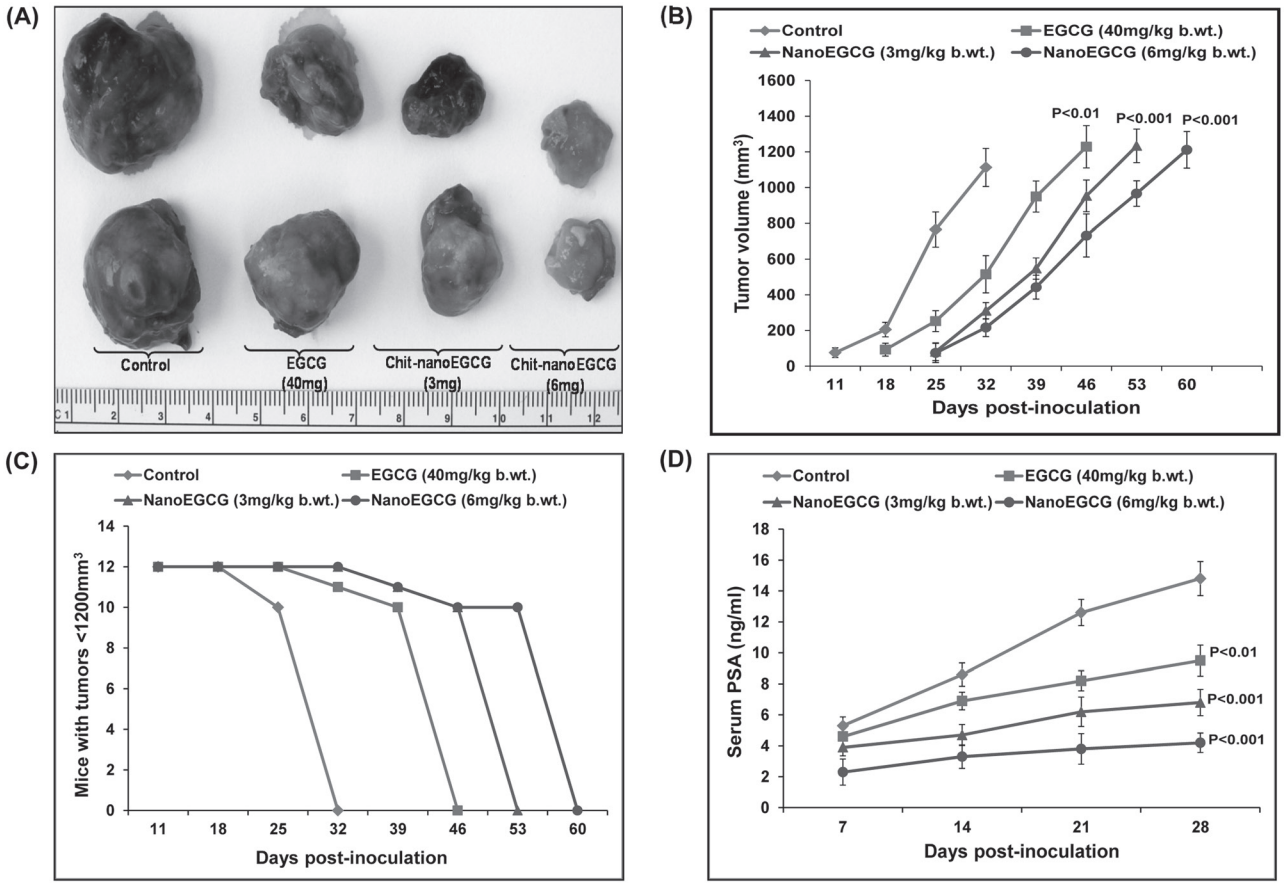

Fig. 3. Effect of Chit-nanoEGCG on PCa tumor growth and serum PSA in athymic nude mice. Forty-eight athymic nude mice were randomly divided into four groups of 12 mice in each group. Approximately 1 million 22Rv1 cells were subcutaneously injected in each flank of mouse to initiate tumor growth. One day after cell implantation, Group I received void chitosan nanoparticles and served as the control. Group II received EGCG (40 mg/kg body wt). Group III received Chit-nanoEGCG (3 mg/kg body wt) and Group IV received Chit-nanoEGCG (6 mg/kg body wt) by oral intubation five times a week. Once tumors started to grow, their sizes were measured weekly and the tumor volume was calculated. (**A**) Photographs of excised tumors from each group once the tumors reached 1200 mm^3 in the control group. (**B**) Average tumor volume of control group, EGCG-treated and Chit-nanoEGCG-treated mice plotted over days after tumor cell inoculation. (**C**) Number of mice remaining with tumor volumes of 1200 mm^3 after they received treatment with EGCG and Chit-nanoEGCG for the indicated days. (**D**) Serum PSA levels were analyzed by ELISA. Details are described in Materials and methods.

and PCa (36). In our study, we found that there was significant inhibition of secreted PSA levels by 13–36%, 26–54% and 57–72% in EGCG, Chit-nanoEGCG (3 mg/kg body wt) and Chit-nanoEGCG (6 mg/kg body wt) treated groups, respectively, compared with the control group normalized to tumor volume (Figure 3D). There was more marked inhibition in serum PSA levels on treatment with high dose of Chit-nanoEGCG, suggesting that less PSA was detected in the serum of these mice. Hence, our results show that treatment of mice with Chit-nanoEGCG caused dose-dependent significant decrease in the serum PSA in athymic nude mice and the effect was more pronounced with Chit-nanoEGCG than with EGCG (Figure 3D).

Cleavage of PARP, effect on Bax, Bcl-2 and induction of active caspases-3, -8 and -9 by Chit-nanoEGCG

PARP is a nuclear protein, which gets activated upon damage to DNA and modify DNA-associated proteins by adding chains of ADP-ribose units (37). As shown by immunoblots in Figure 4A, PARP cleavage analysis showed that the full-size PARP (116 kDa) protein was cleaved 4-fold to yield an 85 kDa fragment on treatment with Chit-nanoEGCG. There was also change in the PARP ratio supporting apoptosis as shown in Figure 4B. The mitochondrial pathway of apoptosis is activated by numerous intracellular and extracellular stress signals, which result in initiation of proapoptotic proteins such as Bax and antiapoptotic proteins such as Bcl-2 (38). There was also a 1.8- to 2.9-fold increase in the protein expression of Bax, whereas the protein expression of Bcl-2 was decreased by 3.6- to 7.8-fold by Chit-nanoEGCG in a dose-dependent fashion (Figure 4A), further confirming the induction of the apoptotic process. Modulations in the expression of Bax and Bcl-2 by Chit-nanoEGCG resulted in a change in the ratio of these molecules in a way that favored apoptosis (data not shown).

Caspases are a family of cysteine proteases, which at their active site contain cysteine residue and cleave their substrate at position next to aspartate residue and are involved in various functions, such as inflammatory, development and apoptotic pathways (39). To investigate the involvement of caspases in Chit-nanoEGCG-mediated apoptosis, we first evaluated the effect of Chit-nanoEGCG on the protein expression of active caspases-3, -8 and -9. We found that there were 2- to 3-fold increase in the activation of caspases-3, -8 and -9 in tumor tissues of mice treated with both doses of Chit-nanoEGCG compared with that seen in group treated with EGCG and control (Figure 4C). The above results indicate that Chit-nanoEGCG treatment of mice resulted in apoptosis via activation of caspases.

Inhibition of cell proliferation markers in tumor tissues of athymic nude mice by Chit-nanoEGCG

Ki-67 protein is involved in regulation of cell cycle and cell proliferation. It is expressed in proliferating cells during all active phases of the cell cycle and is regarded as the most promising biomarker for cell proliferation (40). Proliferating cell nuclear antigen (PCNA) is involved in eukaryotic DNA synthesis and plays an important role in the cell cycle regulation. Its expression is significantly increased

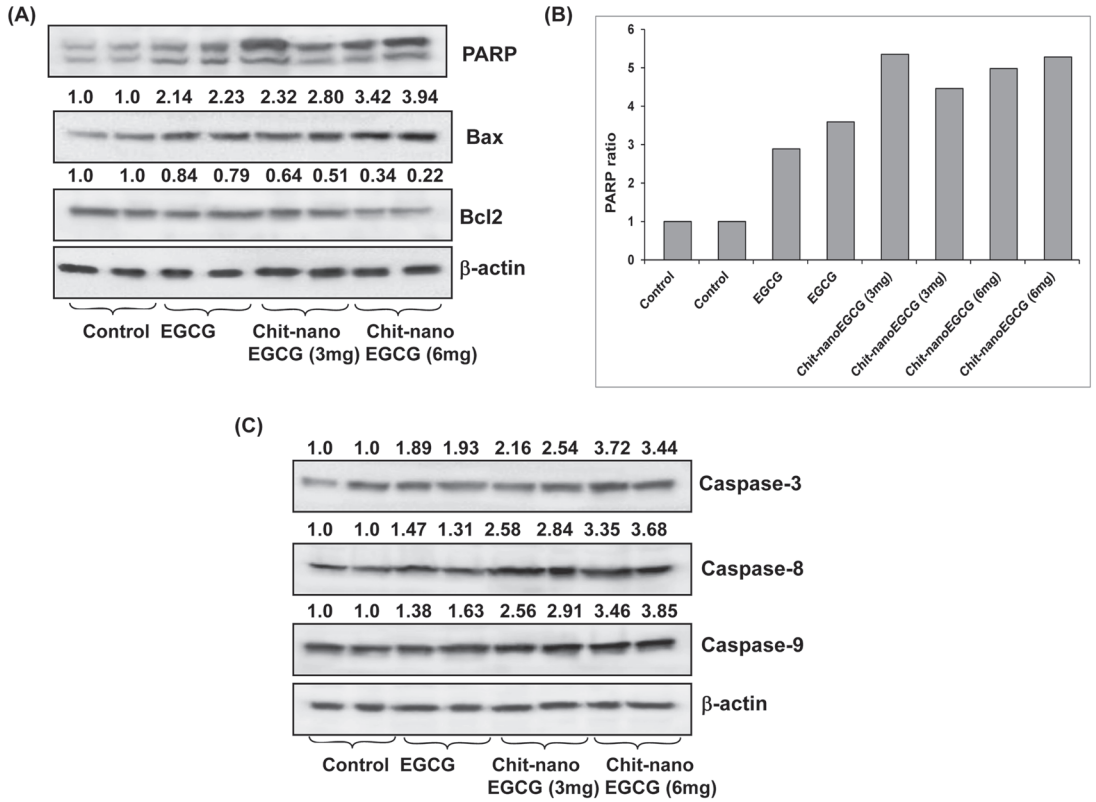

Fig. 4. Effect of Chit-nanoEGCG on PARP, Bax, Bcl-2 and active caspases in tumor tissues of athymic nude mice. (**A**) Effect of Chit-nanoEGCG on levels of PARP cleavage, Bax and Bcl-2 in tumor tissues. (**B**) PARP ratio. (**C**) Effect of Chit-nanoEGCG on protein expression of active caspases in tumor tissues. As detailed in Materials and methods, total tissue lysates were prepared and 40 μg protein was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by immunoblot analysis and chemiluminescence detection. Equal loading of protein was confirmed by stripping the immunoblot and reprobing it for β-actin. The values above the figures represent relative density of the bands normalized to β-actin.

in the process of malignant transformation of normal epithelium and is considered as a cell proliferation marker. We observed that there was 4.1- to 5.9-fold less expression of Ki-67 and 5.5- to 6.8-fold less expression of PCNA in tissues of mice treated with Chit-nanoEGCG than control group (Figure 5A and B). The apparent increase in Ki-67 and PCNA immunostaining-positive cells is a general feature of multistep carcinogenesis. In this study, there was increased cell proliferation activity in tissues of non-lesion areas or lesions of the tumor. In this study, the tumors in mice given Chit-nanoEGCG had lower Ki-67 and PCNA-positive index when compared with EGCG-treated and control groups. The inhibitory effect of Chit-nanoEGCG on the growth of prostate tumors may be due to, in part, modification of cell proliferation.

Inhibition of angiogenesis markers in tumor tissues of athymic nude mice by Chit-nanoEGCG

Angiogenesis is a critical process during tumor development and metastasis is the target of many antitumor therapies (41). The process of angiogenesis involves the activation, proliferation and migration of endothelial cells toward angiogenic stimuli produced by the tumor. CD31 is a transmembrane glycoprotein and a platelet endothelial cell adhesion molecule, which recognizes preexisting and newly formed vasculature regardless of size in normal and PCa tissues with same intensity (42). VEGF is one of the most essential angiogenic factors, which exerts its biological effects by binding to its receptor tyrosine kinases, expressed on endothelial cells (43). By immunohistochemical analysis of the tumor tissue samples from athymic nude mice, we observed numerous CD31 and VEGF-positive cells in tumors from control group. There was 5.7- to 8.2-fold expression of CD31 and 4.1- to 5.9-fold VEGF-positive cells in control group than in Chit-nanoEGCG-treated groups (Figure 6A and B).

Discussion

Green tea has shown potential in laboratory, clinical and epidemiological studies; however, concerns related to bioavailability of its most active component EGCG and perceived toxicity associated with its long-term use affect its clinical outcome (44,45). This study suggests a different approach involving nanoencapsulation of EGCG for oral consumption for the prevention and treatment of PCa. Oral consumption is the most preferred and suitable form of delivery of chemopreventive agents. Though, one disadvantage of using polylactic acid–polyethylene glycol nanoparticles is their unstable nature in acidic environment and therefore is not recommended for oral consumption (46). To overcome this obstacle, we have been successful in developing an oral formulation of nanoEGCG employing a naturally occurring polymer chitosan, which we observed to result in a steady and sustained release of EGCG in the plasma of mice. Chitosan nanoparticles were synthesized in aqueous conditions by promoting the interaction of the NH_2 group present in chitosan with the phosphate group present in TPP. We chose water-soluble chitosan as opposed to the more widely used chitosan that is soluble in mildly acidic pH because using water-soluble chitosan eliminates harsh acidic conditions, which might have adverse effects on the active ingredients. This nanoformulation also has the potential to be used as a carrier system for many of the bioactive compounds that have sensitivity to acidic pH. The size of the nanoparticles can be manipulated by changing parameters like the concentration of chitosan and TPP. However, after extensive experimental studies, we found that this formulation was the optimum formulation with the highest loading efficiency of EGCG. Therefore, we chose this particular method of preparing Chit-nanoEGCG, which can produce a size ~150–200 nm with spherical morphology and with loading efficiency of ~10% w/w to the nanomaterials.

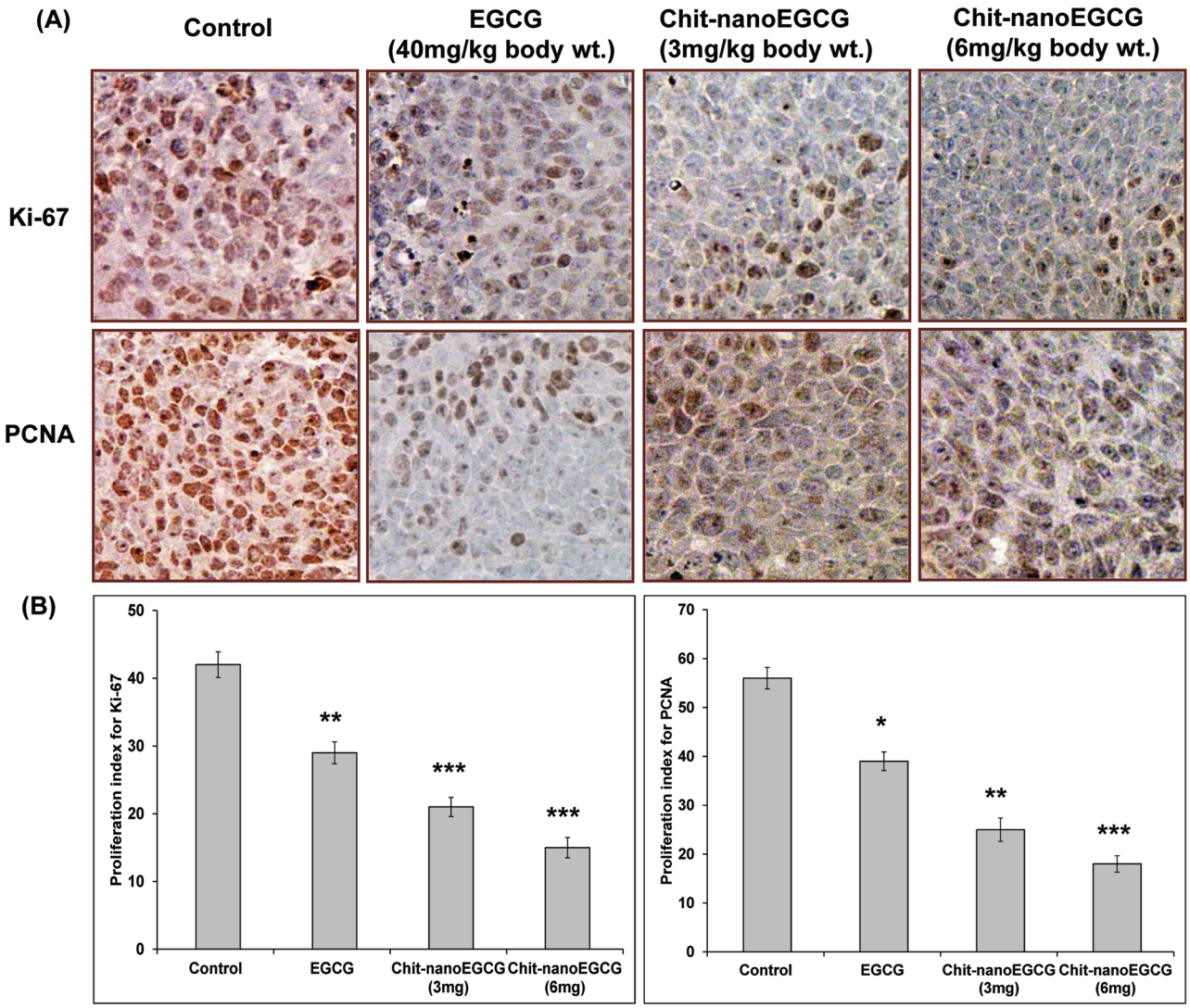

Fig. 5. (**A**) Effect of Chit-nanoEGCG on expression of Ki-67 and PCNA in tumor tissues of athymic nude mice. Tumor sections from athymic nude mice were stained using specific antibodies as detailed in Materials and methods. Counterstaining was performed with hematoxylin. Scale bar, 50 µm. Photomicrographs (magnification, ×20) show representative pictures from two independent samples. (**B**) Proliferation index for Ki-67 (left panel) and PCNA (right panel) is shown. $^{*}P < 0.05$ and $^{**}P < 0.01$ and $^{***}P < 0.001$, versus control group.

Published work suggests that EGCG functions as a chemopreventive as well as chemotherapeutic agent due to its ability to suppress a wide array of biological effects including on many signal transduction pathways (47,48). Several epidemiological studies have shown that that there is low incidence of PCa among Japanese and Chinese populations with a high intake of green tea, which has EGCG as the main constituent (44,49). It is a powerful antioxidant and prevents oxidative damage in healthy cells. EGCG appears to afford protection against PCa through multiple biological mechanisms and by acting on the specific molecular targets and intracellular pathways (44,45). One of the major problems that hinder the use of many natural active ingredients as anticancer drugs (especially EGCG) is the poor absorption in the gastrointestinal tract. In the case of oral delivery of an active biomaterial or natural product, the degradation starts in the stomach, due to the acidic pH. Our chitosan nanoparticles provide a platform for EGCG to be embedded in the polymeric network, and therefore EGCG can be protected from degradation in the harsh conditions of the stomach and the gastrointestinal tract. One of the most important factors for carrier-mediated delivery of drugs through an oral route is that the carrier system must not degrade and release at acidic pH, thus releasing the entire drug. At the same time, it has to retain its capability to release the drug at neutral pH. Our release kinetics experiments strongly support that the release of EGCG in simulated gastric juice was minimal, and at the same time, EGCG was able to be released in a faster way. An initial burst at ~20% is because of the release of the desorbed EGCG from the particle surface, and thereafter the slow release up to 24 h (~50%) resulted from the diffusion of the drug to the aqueous environment, and finally the slow and steady sustained release was because of the diffusion as well as the disintegration of the polymeric nanoparticulate network. The increased EGCG level observed in the blood serum after 3 h compared with free EGCG was perhaps because of the special mucoadhesive properties of the chitosan polymers. Thus, it is postulated that chitosan nanoparticles can increase the retention time of the EGCG by adhering to the mucosal surface in the gastrointestinal tract as well as transiently opening the tight epithelial cellular junctions (50) and thereby increasing the level of EGCG in the blood serum.

The ultimate goal of our nanoformulation Chit-nanoEGCG was to improve the efficacy of EGCG in PCa tumor xenografts. The choice of androgen-refractory 22Rv1 cells was because of their unique properties such as rapid and reproducible tumor growth *in vivo* and secretion of PSA in the bloodstream of the host. Our data clearly established that Chit-nanoEGCG substantially inhibits the growth of PCa even with a 6-fold lower dose of EGCG compared with free EGCG. PSA

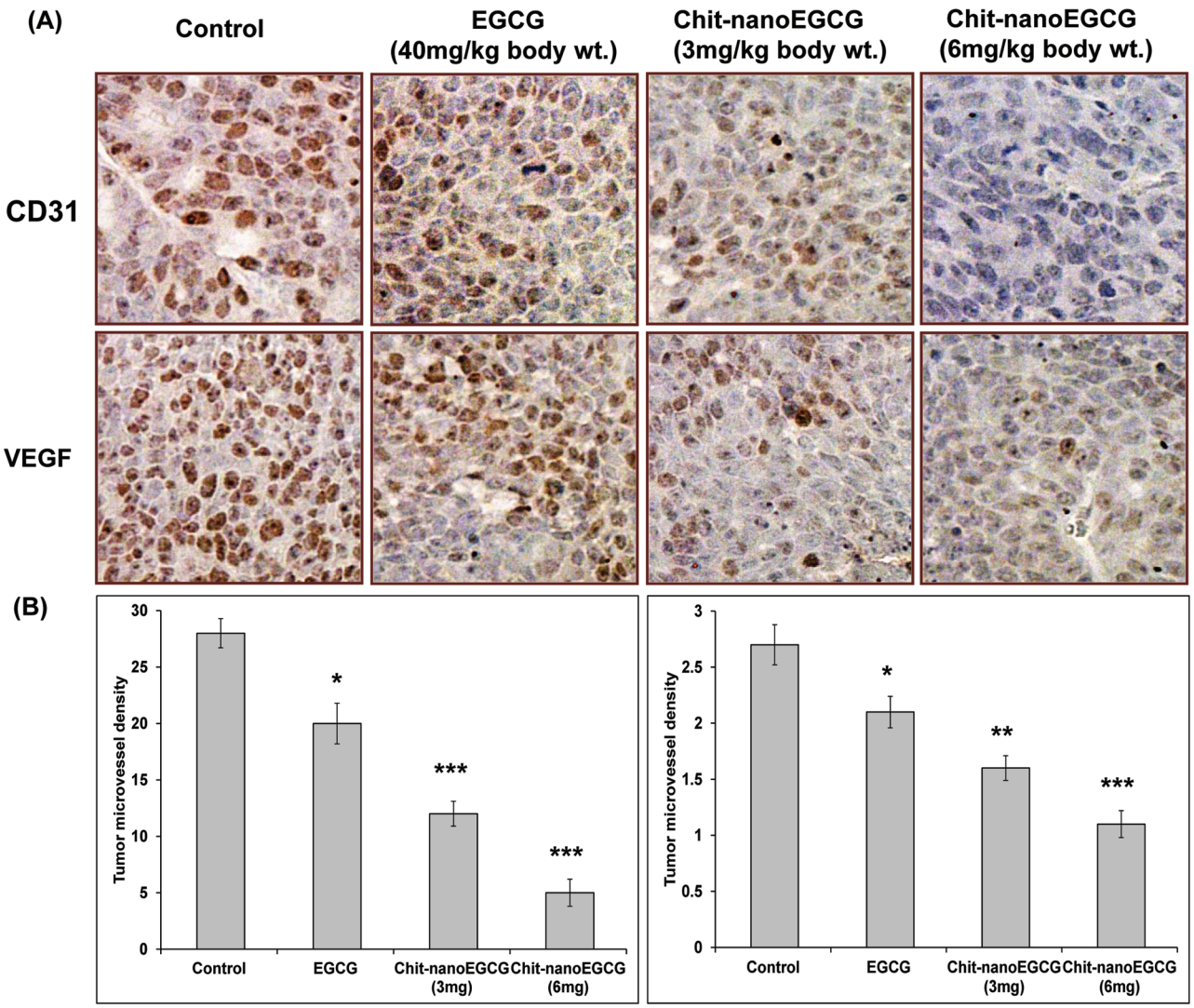

Fig. 6. (**A**) Effect of Chit-nanoEGCG on expression of CD31 and VEGF in tumor tissues of athymic nude mice. Tumor sections from athymic nude mice were stained using specific antibodies as detailed in Materials and methods. Counterstaining was performed with hematoxylin. Scale bar, 50 µm. Photomicrographs (magnification, ×20) show representative pictures from two independent samples. (**B**) Tumor microvessel density (left panel) and VEGF immunoreactivity score (right panel) was scored as 0+ (no staining), 1+ (weak staining), 2+ (moderate staining), 3+ (strong staining) and 4+ (very strong staining). $*P < 0.05$ and $**P < 0.01$ and $***P < 0.001$, versus control group.

is a serine protease with highly prostate-specific expression and is the most widely employed marker in the detection of early PCa. For these reasons, agents that could reduce PSA levels may have important clinical implications for PCa. It is currently the most accepted marker for assessment of PCa progression in humans and is being detected in the serum of patients with prostate diseases including prostatitis and benign prostatic hypertrophy. Our results showed that treatment of athymic nude mice with Chit-nanoEGCG caused a dose-dependent significant decrease in the serum PSA and the effect was more pronounced with Chit-nanoEGCG than with free EGCG.

PARP is involved in several cellular activities such as regulation of transcription and DNA repair (51) and its inhibitors are being investigated in various clinical trials (37,52). The process of apoptosis comprises of the sequential activation of caspases, which are the key players in apoptotic cell death. Our results showed that there was cleavage of PARP, induction of Bax inhibition of Bcl-2 and activation of caspases-3, -8 and -9 in the tumor tissues of mice treated with Chit-nanoEGCG (Figure 5A). There was also decrease in the expression of Ki-67, PCNA (cell proliferation markers), CD31 and VEGF (markers of angiogenesis) in tissues of mice treated with Chit-nanoEGCG than with free EGCG. Although our experiments were performed in a preclinical setting, our results indicate that in the near future, nanotechnology-based, non-invasive platform has tremendous potential to replace many of the invasive techniques (like radiation therapy or surgery) to treat PCa.

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