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Inhibition of Bacterial Growth and Intramniotic Infection in a Guinea Pig Model of Chorioamnionitis Using PAMAM Dendrimers

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Abstract

Dendrimers have emerged as topical microbicides to treat vaginal infections. This study explores the *in-vitro, in-vivo* antimicrobial activity of PAMAM dendrimers, and the associated mechanism. Interestingly, topical cervical application of 500 μg of generation-4 neutral dendrimer (G_4-PAMAM-OH) showed potential to treat the *Escherichia coli* induced ascending uterine infection in guinea pig model of chorioamnionitis. Amniotic fluid collected from different gestational sacs of infected guinea pigs post treatment showed absence of *E. coli* growth in the cultures plated with it. The cytokine level [tumor necrosis factor (TNFα) and interleukin (IL-6 and IL-1β)] in placenta of the G_4-PAMAM-OH treated animals were comparable to those in healthy animals while these were notably high in infected animals. Since, antibacterial activity of amine-terminated PAMAM dendrimers is known, the activity of hydroxyl and carboxylic acid terminated PAMAM dendrimers was compared with it. Though the G_4-PAMAM-NH_2 shows superior antibacterial activity, it was found to be cytotoxic to human cervical epithelial cell line above 10μg / mL, while the G_4-PAMAM-OH was non cytotoxic upto 1mg / mL concentration. Cell integrity, outer (OM) and inner (IM) membrane permeabilization assays showed that G_4-PAMAM-OH dendrimer efficiently changed the OM permeability, while G_4-PAMAM-NH_2 and $G_{3.5}$-PAMAM-COOH damaged both OM and IM causing the bacterial lysis. The possible antibacterial mechanism are; G_4-PAMAM-NH_2 acts as polycation binding to the polyanionic lipopolysaccharide in *E. coli*, the G_4-PAMAM-OH forms hydrogen bonds with the hydrophilic O-antigens in *E. coli* membrane and the $G_{3.5}$-PAMAM-COOH acts as a polyanion, chelating the divalent ions in outer cell membrane of *E. coli*. This is the first study which shows that G_4-PAMAM-OH dendrimer acts as an antibacterial agent.

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Keywords

PAMAM dendrimer; antimicrobial activity; Gram-negative bacteria; cytotoxicity; cell membrane

1. Introduction

Dendrimers are emerging as the new topical antimicrobial agents (Svenson and Tomalia, 2005). Recent developments show that the polylysine dendrimers are effective against the simplex herpes virus (Bourne et al., 2000; Svenson and Tomalia, 2005). The polylysine (SPL7013) dendrimer is currently undergoing Phase I and Phase II human clinical trials to test its efficacy against genital herpes and HIV, safety, tolerability, retention and duration of activity (Halford, 2005; Mumper et al., 2009). Different approaches have been used to enhance the antimicrobial activities employing dendrimers and the most conventional being carriers for the antimicrobial agents, as encapsulating or complexing agents for antibacterial agents, by competitively inhibiting the binding of bacteria to the host cells (Cloninger, 2002; Hou et al., 2009) and by exerting the microbial activity by themselves. Quinolone drugs encapsulated in PAMAM dendrimers were active and useful for topical delivery of microbicides (Cheng Y, 2007). Triazine-based antibiotics encapsulated in the dendrimer beads provided enhanced inhibition of microbial growth when compared to the non-denrimerized triazine antibiotics (Lebreton, 2003). The silver complexes and nanocomposites with PAMAM dendrimer resulted in increased antibacterial activity towards the *S. aureus*, *P. aeruginosa* and *E. coli* due to the improved contact of microbes with the organized silver domains (Balogh, 2001).

Literature reveals that the dendrimer core, the surface charge and functionality, 3D structure and the size of the dendrimer are key factors that affect the antibacterial activity. The PAMAM (polyamidoamine) dendrimer with amine terminations exhibited antibacterial activity against gram negative *E. coli*, *P. aeruginosa* and gram positive *S. aureus* (Calabretta et al., 2007; Lopez et al., 2009). Glycodendrimers are known as antimicrobial agents (Cloninger, 2002). Ortega et al (Ortega, 2008) reported that the amine and ammonium terminated cationic carbosilane dendrimers were more potent towards the gram positive bacteria than the gram negative bacteria. Amine-terminated dendrimers with poly(propyleneoxide) amine core and methylacrylate and ethylenediamine core were found to demonstrate antibacterial and antifungal activities comparatively higher or equipotent to the antibacterial and antifungal agents used conventionally (Tulu et al., 2009). The quaternary ammonium terminated poly(propyleneimine) (PPI) dendrimer were found to be antibacterial against gram negative and gram positive bacteria (Chen et al., 2000; Chen and Cooper, 2002). The mechanism of antibacterial activity of the polycationic PPI dendrimers was attributed to the displacement of the divalent cations from the outer membrane of the gram negative bacteria causing cell wall disruption and lysis. Though the amine-terminated PPI dendrimers show excellent transfection and antibacterial activity, the major limitation to their use is the cytotoxicity and hence surface modification was sought to reduce cytotoxicity (Dutta et al., 2008; Tziveleka et al., 2007; Yang et al., 2008). Considerable effort has been directed in the past towards the surface group modification of the amine-terminated PAMAM dendrimers for reduce the cytotoxicity (Jevprasesphant et al., 2003; Kim et al., 2008; Kolhatkar et al., 2007; Yang et al., 2008). Size of the dendrimer affects its ability to penetrate the bacterial cell and therefore its antibacterial activity. For example, G_3 PAMAM dendrimers were more effective antibacterial agents than G_5 PAMAM dendrimers (Lopez et al., 2009). Similarly, lower generation carbosilane dendrimers were found to be more effective than higher generation dendrimers (Ortega, 2008). Typically, dendrimers have been investigated as carriers for drugs and imaging agents, but these recent findings

present the perspective of dendrimers as new antimicrobial agents (Svenson and Tomalia, 2005).

Efforts continue to identify the newer and better antimicrobial agents. Antibacterial activity of amine-terminated PAMAM dendrimers is well recognized (Calabretta et al., 2007; Lopez et al., 2009), to the best of our knowledge, the assessment of the antibacterial activity of carboxylic and hydroxyl terminated PAMAM dendrimers has not been previously reported. In the present study, we have evaluated mechanisms by which hydroxyl and carboxylic acid terminated PAMAM dendrimers affect the cell wall of *E. coli*. Interestingly, we found that the hydroxyl terminated PAMAM dendrimer exhibited antibacterial activity *in-vitro*. We evaluated the ability of the hydroxyl terminated PAMAM dendrimer to treat the *E. coli* induced uterine infection *in-vivo* in pregnant guinea pig. Since, the guinea pig model of chorioamnionitis is well established to demonstrate the injury caused to the fetus resulting from *E. coli* infections (Patrick et al., 2004), we used this model for evaluating the *in-vivo* activity of G4-PAMAM-OH.

Intrauterine infection is usually caused by microorganisms ascending from vaginal and affecting the fetus and amniotic fluid leading to chorioamnionitis, cerebral palsy, increased efficiency of HIV seroconversion, miscarriage, and spontaneous preterm birth (Chaim et al., 1997),(Romero, 2003; Ugwumadu, 2007). Chorioamnionitis is known to cause fetal brain injury (Patrick et al., 2004) due to the generation of pro-inflammatory cytokines (Dickinson et al., 2009; Harnett et al., 2007). Antibacterial and antifungal agents are applied to vagina and cervix to treat intrauterine infections in the pregnant women (Chaim et al., 1997; Ugwumadu, 2007). In the present study we show that *E. coli* infection in pregnant guinea pig can be treated by topical vaginal and cervical application of G_4-PAMAM-OH dendrimer. This is the first report using the guinea pig model of chorioamnionitis to induce *E. coli* infections and show the effective inhibition of bacterial growth by treatment with G_4-PAMAM-OH. Our results show that the cytokine levels in placenta of the G_4-PAMAM-OH treated animals were comparable to those in healthy animals and significantly less than infected animals. Although PAMAM dendrimers are the most extensively studied dendrimers the antimicrobial activity of unmodified G_4-PAMAM-OH and $G_{3.5}$-PAMAM-COOH has not been reported previously. Though G_4-PAMAM-NH_2 dendrimer shows strong antibacterial activity it shows high cytotoxicity to human cervical cell line and the antibacterial activity of G4-PAMAM-OH dendrimer is notable since it is non-cytotoxic at higher concentrations. Our findings bring out that G_4-PAMAM-OH has a potential as antibacterial agent.

2. Experimental Section

2.1. Materials

The PAMAM dendrimers (generation 4, with end groups OH, NH_2 and generation 3.5 COOH 14.93 % w/w in methanol) were purchased from Dendritech. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), 2-Nitrophenyl-β-D-galactopyranoside (ONPG), Osmium tetrozxide N-Phenyl-1-naphthylamine (NPN), glutaraldehyde and hexamethyldisilazane were purchased from Invitrogen. Nutrient broth and nutrient agar were purchased from BD Biosciences. Mouse TNFα, IL-6 and IL-1β ELISA kits were purchased from R&D Systems.

2.2. Preparation of bacteria

Escherichia coli (ATCC 11775) isolated from human urine is the bacterial strain used in this study. Single colony on nutrient agar was used to inoculate 5 mL of nutrient broth at 37 °C overnight. A small volume (100 μL) of this growth was used to inoculate 20 mL of nutrient

broth media at 37 °C for 6 h. The bacteria were resuspended at 10^6 colony forming units (CFU)/mL for the experiments.

2.3. Bacterial growth inhibition assays

The inhibitory concentration (IC_{50}) of dendrimers was determined using the broth microdilution method (Lopez et al., 2009; Wiegand et al., 2008). Briefly, serial dilutions of dendrimers (0.76g/mL to 200 mg/mL) were prepared in PBS and combined 1:1 v/v with bacteria at 10^6 CFU/mL in a 96 well polypropylene plate. After incubation at 37 °C for 18 h, the absorbance was measured at 650 nm using a microplate reader to assess the cell growth. The positive-control wells contained PBS and nutrient broth medium inoculated with bacteria (5×10^5 CFU/mL), and the negative-control wells contained PBS and nutrient broth medium without bacteria. The IC_{50} value was determined as the concentration of the dendrimers which inhibits 50% of microbial growth after 18–24 h incubation (Lopez et al., 2009; Wiegand et al., 2008). The % survival of the bacteria was determined on the basis of the positive control which was considered as 100 %.

2.4. Morphology of the bacteria

Scanning Electron Microscopy (SEM) analyses were performed to investigate the morphological changes of the *E. coli* following 8h treatment with dendrimers G_4-PAMAM-OH, $G_{3.5}$-PAMAM-COOH and G_4-PAMAM-NH_2 at concentrations arrived at from bacterial growth inhibition assays. *E. coli* were fixed with 2.5 % glutaraldehyde for 2 h. The samples were washed thrice with deionised water, and postfixed with 1% osmium tetraoxide for 2 h, followed by washing thrice with deionised water. The samples were dehydrated with a series of ethanol solutions (50 %, 70 %, 90 % and 100 %) followed by 10 min in 100 % hexamethyldisilazane (HMDS), and were air dried. The samples were coated with gold and observed under SEM (HITACHI S-2400 Scanning Electron Microscope) at 20 kV.

2.5. Cell Integrity

The bacterial cell membrane integrity was examined by determination of the release of nuclear acids (RNA/DNA) material absorbing at 260 nm (Je and Kim, 2006b; Ibrahim, 1991). *E. coli* were harvested, washed and resuspended in 0.5% NaCl solution with an absorbance of 0.7 at 420 nm. 150 μL of G_4-PAMAM-OH, $G_{3.5}$-PAMAM-COOH and G_4-PAMAM-NH_2 solutions at concentrations arrived at from bacterial growth inhibition assays were mixed with 150μL of bacterial suspension in a 96-well plate, control was carried out with 0.5% NaCl alone, and the release over time of nuclear materials was monitored with a UV spectrometer by measuring the absorbance at 260 nm.

2.6. Outer Membrane (OM) Permeabilization Assay

The OM permeabilization of *E. coli* by dendrimers was evaluated using the hydrophobic NPN (1-N-phenylnaphthylamine) fluorescent probe (Helander and Mattila-Sandholm, 2000; Je and Kim, 2006a). *E. coli* were harvested, washed, and resuspended in 0.5% NaCl solution with an absorbance of 1.0 at 420 nm. The concentration of dendrimers was the same as described in previous section. 100 μL of *E. coli* and 50 μL of dendrimers were mixed with 50 μL of 40 μM NPN in black fluorotiter plate, control was carried out with 0.5% NaCl alone. An increase in fluorescence due to partitioning of NPN into the OM was recorded as a function of time until no further increase in intensity was observed. Excitation and emission wavelengths were set at 350 and 420 nm, respectively.

2.7. Inner Membrane (IM) Permeabilization Assay

IM permeabilization was determined by measuring the release of cytoplasmic β-galactosidase activity from *E. coli* into the culture medium using ONPG as the substrate (Je

and Kim, 2006b). *E. coli* were harvested, washed and resuspended in 0.5% NaCl solution an absorbance of 1.2 at 420 nm, then re-suspended in half volume of 0.5% NaCl solution. The concentration of dendrimers was the same as described in previous section. 100 μL of *E. coli* and 100 μL of dendrimers were mixed with 10 μL of ONPG (30 mM) in a 96-well plate, control was carried out with 0.5% NaCl alone. The production of *0*-nitrophenol over time was determined by monitoring the change in absorbance at 420 nm using a spectrophotometer.

2.8. Cell culture

Human cervical epithelial End1/E6E7 cells were obtained from ATCC, maintained in DMEM/F-12K medium supplemented with 20 % fetal bovine serum (FBS). Mouse microglial BV-2 cells were obtained from Children's Hospital of Michigan Cell Culture Facility and maintained in DMEM supplemented with 5 % FBS. These cells were cultured at 37 °C with 5 % CO_2 and the media was replaced at 2 days interval.

2.9. Evaluation of normal cell cytotoxicity

End1/E6E7 and BV-2 (passage 19) cells were seeded into a 96-well plate at 1.5×10^4 / well, and 5×10^3 /well, respectively. After 24 h, cells were exposed to various concentrations of dendrimers (10 ng/mL to 1 mg/mL) in serum free medium for 24 h. Controls were carried out with medium alone. Cytotoxic effect was determined using MTT assay. The proportion of viable cells in the treated group was compared to that of the control.

2.10. Flow Cytometry Analysis

E. coli (10^6 CFU) and BV-2 (10^6) cells were treated with 10 μg/mL of FITC-labeled G_4-PAMAM-OH dendrimer. The FITC-labeled G_4-PAMAM-OH dendrimer was prepared as previously reported (Kolhe et al., 2006). The *E. coli* and cells were washed 3 times with PBS, resuspended in 1% formaldehyde, and analyzed using a flow cytometer (FACS caliber, Becton Dickinson) by counting 20,000 and 10,000 events, respectively. The mean fluorescence intensity of cells was calculated using the histogram plot.

2.11. Confocal Laser Scanning Microscopy

Microglial cells (10^6) were treated for 2 h with 10 μg/mL of G_4-PAMAM-OH-FITC. The cells were washed with PBS three times and fixed with 4 % para-formaldehyde for 20 min. Images were captured using a confocal microscope (Zeiss LSM 310) using a magnification of 400×. The excitation and emission wavelengths were 488 and 518 nm respectively, for FITC.

2.12. Evaluation the antimicrobial activity in guinea pig model of chorioamnionitis

All the animal experimental procedures were approved by the institutional animal care and use committee of Wayne State University. Intracervical bacterial inoculation was performed as previously reported (Patrick et al., 2004). Briefly, pregnant Dunkin-Hartley strain guinea pigs (Charles River) at 52 days of gestation were anesthetized with 1.5% isoflurane using the mask. An endoscope was used to visualize the cervix. Guinea pigs were inoculated intra-cervically with 150 CFU *E. coli* (n=11) to induce infection. Dendrimer G_4-PAMAM-OH 500 μg was injected into the cervix 5 min after *E. coli* inoculation in the treatment group 3 (n=4). The *E. coli* inoculated guinea pigs without treatment (group 2) were used as positive control (n=4). The guinea pigs without any treatment (group 1) and inoculation were used as negative controls (n=3). Forty eight hours after intervention, guinea pigs were euthanized with pentobarbital sodium (120 mg/kg) and midline laparotomy was performed to expose uterus. Amniotic fluid was collected from each gestational sac and 50 μL was plated on nutrient agar to determine the presence of microbiologic chorioamnionitis.

2.13. Cytokine quantification in placenta

The placental tissue (0.3 g) was homogenized in 1 mL RIPA lysis buffer. The homogenate was kept on ice for 30 min, centrifuged at 10,000 g for 25 min at 4°C and the protein concentration of supernatant was determined. Cytokines; tumor necrosis factor (TNFα), interleukin (IL-6 and IL-1β) concentrations were measured in the total protein fraction using ELISA kits (Ethier-Chiasson, 2008).

2.13. Statistical analysis

Data are presented as mean ± SD. Specific comparisons between control and individual experiment were analyzed by ANOVA test with *p*-value less than 0.05 considered as statistically significant.

3. Results

3.1. Antimicrobial Assay

We used the antibacterial assay procedure reported previously (Lopez et al., 2009) to assess the antimicrobial activity of G_4-PAMAM-OH and $G_{3.5}$-PAMAM-COOH dendrimers towards the gram negative bacteria *E. coli* and compared it with the activity of G_4-PAMAM-NH_2. We used *E. coli* in this study since it is known to cause the chorioamnionitis in pregnancy, in an established guinea pig model based on *E. coli* infection (Patrick et al., 2004). We have used *E. coli* for *in-vitro* and *in-vivo* evaluations to demonstrate the antibacterial activity of PAMAM dendrimers. In the present study we have measured the IC_{50} values of PAMAM dendrimers using a modified broth microdilution assay in a 96-well plate format. The optical density of the suspension of bacteria in different dendrimer solutions was measured at 650 nm. The IC_{50} value of the dendrimer was then obtained from the plot of % survival of bacteria *vs*. the concentrations of the dendrimer and the plot of optical densities *vs*. the concentrations of the dendrimer. G_4-PAMAM-OH, $G_{3.5}$-PAMAM-COOH and G_4-PAMAM-NH_2, dendrimers inhibited the growth of *E. coli* in a concentration-dependent manner as seen from 18 h treatment (Fig. 1). The strong antimicrobial activity of G_4-PAMAM-NH_2 is consistent with that reported previously (Calabretta et al., 2007). It is interesting to note that G_4-PAMAM-OH markedly inhibited the growth of *E. coli* from 3.13 mg/mL to 25.0 mg/mL concentration. $G_{3.5}$-PAMAM-COOH also inhibited the growth of *E. coli* but at relatively higher concentrations 6.25 mg/mL to 100 mg/mL. For our set of conditions we observed the IC_{50} values for G_4-PAMAM-OH, $G_{3.5}$-PAMAM-COOH and G_4-PAMAM-NH_2 as 5.4 mg/mL, 22.0 mg/mL and 3.8μg/mL respectively. Since G_4-PAMAM-NH_2 dendrimer exhibits high cytotoxicity, the G_4-PAMAM-OH was considered for *in-vivo* evaluations in guinea pigs.

For the amine-terminated PAMAM dendrimers, it is proposed that the amino groups form nanoscale holes in supported lipid bilayers of bacterial membrane causing its rupture and cell lysis (Calabretta et al., 2007; Hong et al., 2006; Mecke et al., 2005; Milovic et al., 2005). The quaternary ammonium dendrimers adsorb onto negatively charged bacterial cell surfaces, diffuse through the cell wall, bind to cytoplasmic membrane, disrupt and disintegrate the cytoplasmic membrane, release of electrolytes such as potassium ions and phosphate from the cell and release nucleic materials such as DNA and RNA, all contributing to the death of the bacterial cell (Chen et al., 2000). These reports suggest that dendrimers mediate their antimicrobial activity by disrupting the bacterial outer and inner membrane. The antibiotic ampicillin is known to penetrate the outer membrane of gram negative bacteria and inhibits the bacterial cell wall synthesis. The antibacterial activity of dendrimers is limited to its effect on bacterial membrane permeabilities. However, there has not been any experimental data to support this suggestion and we performed the outer and inner membrane permeabilization assays to gain insights into the bactericidal mechanism of

action of the PAMAM dendrimers. This is discuss0ed in the subsequent sections in the manuscript.

3.2. SEM analysis of the E. coli

SEM studies were performed to examine if dendrimers damage the cell wall of *E. coli*. The concentration representing the IC_{50} values of dendrimers was used. The SEM images collected after 8 h of treatment with dendrimers showed a marked difference in morphology (Fig. 2). The untreated *E. coli* (Fig. 2A) shows distinct rod shape with the intact cell wall. The treatment of *E. coli* with $G_{3.5}$-PAMAM-COOH (Fig. 2B), G_4-PAMAM-OH (Fig. 2C) and G_4-PAMAM-NH_2 (Fig. 2D) shows shrinking, significant disruption of cell walls, and erosion of cell membrane. The major morphological changes observed by SEM support the antibacterial activity obtained in previous section. These studies indicated that major changes in the bacterial cell wall were induced by the dendrimers at the IC_{50} concentrations, indicative of their antibacterial potential. In order to understand the underlying mechanism for the possible damage to the bacterial cell wall, the cell integrity, outer and inner membrane permeabilization studies were conducted.

3.3. Cell Integrity

The cytoplasmic bacterial cell membrane undoubtedly is the target for many antimicrobial agents. The release of intracellular components is a good indicator of the compromise in cytoplasmic membrane integrity of gram negative bacteria resulting from the microbicidal activity of the tested compounds. In the present study, *E. coli* suspensions were treated with G_4-PAMAM-OH, $G_{3.5}$-PAMAM-COOH and G_4-PAMAM-NH_2 dendrimers at the IC_{50} concentrations and the absorbance of the suspensions was recorded at 260 nm. The absorbance significantly increased up to 20 min for each dendrimer at the tested concentration and thereafter the absorbance was almost unchanged (Fig. 3). The increase in the absorbance at 260 nm is due to the leaching of the small ions such as potassium and phosphate, but largely it's attributed to the large nucleotides; DNA and the RNA released from the compromised bacterial cell. Similar observation was reported previously (Chen and Cooper, 2002) for QAC-functionalized dendrimers which exhibited fast killing kinetics till 10 min for *E. coli*. The increased absorbance is an indicator that at tested concentrations the three dendrimers were capable of altering the cytoplasmic membrane (outer cell membrane and cell wall) integrity for the gram negative bacteria.

3.4. Outer Membrane (OM) permeabilization

The outer membrane in the gram negative bacteria acts as a strong barrier to foreign materials such as drugs. When the bacterial outer membrane is intact, it is impervious to the hydrophobic probe NPN. However once the outer membrane is compromised the NPN partitions in the perturbed outer membrane. Hence NPN is used to determine the changes induced by the biocides in the outer cell membrane. Typically, the greater the damage induced on the membrane, higher is the fluorescence reading, meaning greater is the NPN partitioning in membrane (Je and Kim, 2006b). The *E. coli* was treated with solutions of dendrimers as described in previous section. The G_4-PAMAM-NH_2 dendrimer with amine groups on surface showed the highest fluorescence indicative of the rapid and bulk changes induced in the outer membrane of *E. coli* (Fig. 4). The divalent ions; calcium and magnesium play a key role in the stabilization of the bacterial membrane and are electrostatically bound to the anionic lipopolysaccharides (Chen and Cooper, 2002). The displacement of these divalent cations from the phospholipid membrane structure results in the structural instability, causing the disruption and eventually leading to cell death. At physiological pH, all of the primary amines and half of the tertiary amines in the dendrimer core of G_4-PAMAM-NH_2 are protonated (Cakara, 2007; Cakara, 2003). The G_4-PAMAM-NH_2 dendrimer is a polycation and it possibly replaces the divalent ions binding to the

polyanions present in the cell membrane. This could lead to the insoluble polyanion-polycation complex and efflux of the calcium ions both contributing to major structural changes in outer cell membrane resulting in bacterial cell death.

It is interesting to note that the hydroxyl and carboxylic acid terminated dendrimers also showed an increase in the fluorescent intensity suggesting the NPN partitioning in treated *E. coli* (Fig. 4). These results clearly indicate that both G_4-PAMAM-OH and $G_{3.5}$-PAMAM-COOH alter the outer membrane structure of the *E. coli*. The underlying mechanism by which these dendrimers induce changes in the OM needs to be investigated. Several approaches can be considered to explain how the hydroxyl terminated dendrimer might act on the OM of bacteria. The microscopic protonation mechanism showed that at pH 7.4 and 7.5 the tertiary amines in the core of PAMAM dendrimers are protonated (Cakara, 2007;Cakara, 2003). The number of tertiary amines in the core is abundant when compared to the number of surface groups. Hence at pH 7.4, the core tertiary amines of G_4-PAMAM-OH dendrimer are protonated and the surface hydroxyls are expected to bear negative charge and behave as anions. Anions are known to be bactericidal (Ellison et al., 1988;Khan M. A. S, 2000). This could be one of the possible mechanisms by which G4-PAMAM-OH dendrimer affects the E. coli cell wall permeability. Further, the outer membrane of gram negative bacteria is extremely hydrophilic (Benz, 1988). The permeability of gram negative bacterial cell is altered by interaction of its O-antigens (outer polysaccharide) with hydroxyl groups and surface bound water (Jucker, 1997;Jucker, 1998). The lipopolysaccharide of *E. coli forms* with hydroxyls and amines in the chitosan forming a complex which contributes to bactericidal effect of chitosan (Naberezhnykh et al., 2008). The lipopolysaccharide from *E. coli* forms ionic, electrostatic and hydrogen bonds with chitosan and the hydroxyl groups of chitosan act as proton donors contributing towards these interactions. Further, the antibacterial activity due to hydroxyl groups is well established (Aslama, 2009;Myers, 1928). The literature clearly reveals the ability of the outer membrane of gram negative bacteria to form hydrogen bonds with hydroxyl groups. Our SEM images and OM permeability assay measurements together show the changes brought out in the bacterial cell membrane due to the activity of G4-PAMAM-OH dendrimer. Based on experimental and the inferential evidence, one can interpret that G_4-PAMAM-OH dendrimer forms hydrogen bonds with the outer membrane of *E. coli*, another possible mechanism by which it could brings the permeability changes. The in-depth understanding on the mechanism by which G_4-PAMAM-OH works is required and it is beyond the scope of this paper.

At physiological pH, the carboxylic acid terminated dendrimer acts as polyanion and chelates the positive calcium and magnesium ions in the outer membrane altering its structure and permeability. The phosphate group in the phosphatidyl choline (lipid) layers of bacteria has free carboxyl groups and phosphate groups bearing a negative charge which bind to cations. The outer membrane of gram negative bacteria is stabilized by the ionic interaction and binding of the positive ions (calcium and magnesium) to the polar anionic residues on the lipid head groups. This complex is reversible. Since this complex is reversible, the carboxylic groups from dendrimer replace the carboxylic groups from the lipids and bind to positive calcium ions destabilizing the membrane. This could be a possible mechanism by which the $G_{3.5}$-PAMAM-COOH alters the outer membrane of the *E. coli*. There are reports which support this assumption. The ethylenediamine tetraacetic acid is antibacterial as it binds to the membrane stabilizing cations and releases of lipopolysaccharides (LPS)(Ellison et al., 1988). Incorporation of palmitic acid residues in lysozyme rendered it bactericidal to *E. coli as h*ydrophobic interactions occur between the two in LPS zone (Ibrahim, 1991). The anionic phosvitin shows antibacterial activity towards *E. coli* by chelating calcium ions in outer membrane (Khan M. A. S, 2000). Further, anionic peptide MDpep5 acts as antimicrobial by binding to the bacteria through hydrophobic interactions causing disorders in bilayer structure. The membrane disruption is not complete

for anionic MDpep5 when compared to the cationic (AMPs) antimicrobial peptide (Tang et al., 2008). Based on the literature, when we review the results of present study, it appears that at physiological pH the carboxylate ions on the $G_{3.5}$-PAMAM-COOH act as polyanions interacting with the calcium ions in the outer membrane of gram negative bacteria affecting its permeability. As compared to the anionic $G_{3.5}$-PAMAM-COOH, the cationic G_4-PAMAM-NH_2 causes more damage to outer membrane structure, which is again consistent with the observations reported by Tang et al (Tang et al., 2008). From our studies we found that PAMAM dendrimers with amine, hydroxyl and carboxyl terminal group, could each show a different path for interacting with the bacterial outer membrane.

3.5. IM permeabilization

Destabilization of the outer membrane in bacteria is essential for establishing contact with the inner membrane (Benz, 1988; Je and Kim, 2006b). The inner layer of bacteria consists of phosphatidyl glycerol and cardiolipin (Je and Kim, 2006a). The cytoplasmic β-galactosidase is released as a consequence of alteration / compromise in inner membrane and it reacts with ONPG to yield ortho-nitrophenol which is absorbed at 420 nm. The concentrations of three dendrimers were the same as IC_{50} values. There was a lag of 20 min followed by which a slow release of cytoplasmic β-galactosidase was observed for all the dendrimers tested. The release of cytoplasmic β-galactosidase was found to be time dependant (Fig. 5). Amongst the dendrimers evaluated, the G_4-PAMAM-NH_2 showed a marked increase in OD values indicative its capability to induce major permeability changes in inner membrane. The $G_{3.5}$-PAMAM-COOH dendrimer treated bacteria showed increase in OD with respect to time, again indicating the changes brought in the structure of inner wall. The OD values were low for G_4-PAMAM-OH dendrimer as compared to the other dendrimers but they increased slowly over time as seen from Fig. 5. These results indicate that the G_4-PAMAM-OH dendrimer seems to induce profound permeability changes in outer membrane as compared to the inner membrane. While the $G_{3.5}$-PAMAM-COOH dendrimer appears to release the cell intracellular nucleotides more than the other dendrimers, the cationic G_4-PAMAM-NH_2 dendrimer appears to disrupt the IM and OM to a significantly higher extent compared to the other dendrimers.

3.6. Cytotoxicity assay

The cytotoxicity of PAMAM dendrimers was evaluated against human cervical epithelial (End1/ E6E7) and immune cells; mouse microglial cells (BV-2). MTT assay showed that G_4-PAMAM-OH and $G_{3.5}$-PAMAM-COOH dendrimers were non cytotoxic to End1/E6E7 cells and BV-2 cells in 24 h treatment at concentrations 10 ng / mL - 1 mg / mL (Fig. 6). The G_4-PAMAM-NH_2 showed high cytotoxicity above 10 μg / mL concentration to human cervical epithelial End1/E6E7 cells. Also the G_4-PAMAM-NH_2 exhibited cytotoxicity at 1mg / mL concentration to microglial cells. On the basis of the MTT assay, G_4-PAMAM-OH did not exhibit cytotoxicity upto 1mg / mL concentration, while the G_4-PAMAM-NH_2 was found to be cytotoxic at higher concentrations. *E.coli* infections in the vagina can lead to preterm birth. The experimental data shows that G_4-PAMAM-OH dendrimer is non cytotoxic to the human cervical cell line and also exhibits antibacterial activity towards *E. coli*, hence it was chosen as antibacterial agent to prevent preterm birth in pregnant guinea pigs. In our in-vivo experiments we applied a total of 500 μg of G_4-PAMAM-OH to the cervix of *E. coli* infected pregnant guinea pigs and at this concentration the dendrimer showed efficacy.

3.7. Cell entry dynamics of dendrimer in E. coli and microglial cells

The size of the dendrimer affects its antibacterial activity and G_5-PAMAM-NH_2 was less bactericidal than G_3-PAMAM-NH_2 which could penetrate the bacterial cell because of relatively smaller size (Lopez et al., 2009). In the present study, we have evaluated the

generation-4 PAMAM dendrimers and hence the ability of G_4-PAMAM-OH to enter the cells was determined using a FITC tagged G_4-PAMAM-OH-FITC conjugate. The *E. coli* did not show significant increase in fluorescence intensity after treatment with G_4-PAMAM-OH-FITC for 2 h. However, a significant increase in fluorescence intensity was observed after 3 h (Fig. 7A) confirming a slow entry of G_4-PAMAM-OH-FITC conjugate into the *E. coli*. This demonstrates that G_4-PAMAM-OH enters the *E. coli* effectively while the exact location of G_4-PAMAM-OH in the bacteria remains unclear and needs to be further elucidated using other methods. These results support the previous studies where the G_4-PAMAM-OH shows changes in the permeability of the bacterial membrane. The microglial cells showed a significant fluorescence intensity increase within 15 min, as evidenced by a two-order of magnitude increase in the fluorescence intensity. This was followed by a moderate increase in fluorescence over the next 4 h (Fig. 7B) indicating that G_4-PAMAM-OH-FITC conjugate entered the microglial cells. In case of BV-2 cells the cellular entry was visualized by using confocal microscopy showing the localization of G_4-PAMAM-OH-FITC in cytoplasm (Fig. 7C). The uptake of the dendrimer by *E. coli* was slower than that in microglial cells. The difference in the structure of cell membrane may result in different cell entry dynamics of dendrimer in *E. coli* and microglial cells.

3.8. Antimicrobial activity in guinea pig model of chorioamnionitis

The *in-vitro* studies brought out antibacterial potential of G_4-PAMAM-OH dendrimer as seen from the antibacterial assay, OM and IM permeabilization assays and bulk changes in morphology seen from SEM analysis. These interesting results coupled with its non cytotoxicity to human cervical epithelial cells encouraged the evaluation of G_4-PAMAM-OH as an antibacterial agent *in-vivo* using the guinea pig model of chorioamnionitis. Though this model is established for creating infection and assessing injury to the fetus, it has not been previously used to demonstrate the effective treatment. This is the first study to our knowledge, which shows the treatment of the pregnant guinea pig using the model of chorioamnionitis. The ascending *E. coli* infection causes chorioamnionitis which is associated with development of cerebral palsy, a motor disorder in children due to stimulation of proinflammatory cytokines causing white matter damage and fetal brain injury (Patrick et al., 2004). We optimized the dose of the *E. coli* inoculation in the guinea pigs (n=17) in the pilot experiments for the strain (ATCC 11775). We observed that 1000 CFU of *E. coli* effectively induced the infection causing extreme sickness in mother, leading to abortion of dead fetuses within 48 to 72 h. The lower CFU of *E. coli* were subsequently inoculated to identify the optimum dose which lead to infection and yet the guinea pigs did not abort upto 48 h. Based on this evaluation a dose of 150 CFU of *E. coli* was found to effectively induce the infection in the pregnant guinea pigs without leading to abortion of fetuses.

In the present study, chorioamnionitis was induced after intracervical inoculation with *E. coli* in 8 guinea pigs of which n=4 were considered as positive control (group 2) and n=4 were used for treatment with G_4-PAMAM-OH (group 3). None of the amniotic fluid samples plated from the negative control group-1 (n=3) showed evidence of microbiologic chorioamnionitis. Of the pregnant guinea pigs (group 2, n=4) that were inoculated with 150 CFU of *E. coli*, 57.1% of the amniotic fluid samples for different fetus were positive with bacterial growth (indicative of induced infection) as seen from the culture inoculated with it (see Table 1). Prenatal exposure to maternal infection alters cytokine expression in the placenta (Urakuboa, 2001). Abundance of cytokines in placental tissues is an indicator of activation of inflammatory response in gestational membranes with term and preterm parturition (Keelan et al., 1999). The cytokine IL-6 is known to peak after 48 h of infection (Dickinson et al., 2009) and hence in present study animals were sacrificed after 48 h to determine the cytokine level in positive and treated animals. When we compared the

expression of cytokine levels in negative control *vs* the positive controls, the cytokines, especially TNFα and IL-6, increased significantly in the placenta of positive controls after 48 h of inoculation with *E. coli* (Fig. 8). These results demonstrated that chorioamnionitis was successfully induced after 48 h of cervical inoculation with 150 CFU of *E. coli*.

The G_4-PAMAM-OH dendrimer was applied topically at a dose of (625 μg/kg) on the cervical endometrium of guinea pigs (group-3, n = 4) in form of aqueous solution in saline after *E. coli* inoculation. The total amount applied was 500μg dissolved in saline based on the average weight of the guinea pigs (800 g / animal). The amniotic fluid samples for different fetus were collected after 48 h and were plated on the culture plates and evaluated for the bacterial growth. All these samples did not show any bacterial growth (0 %) on the culture plates (Table 1). The study shows that the treatment with G_4-PAMAM-OH dendrimer completely eliminated the bacterial growth and prevented bacteria ascension into uterine cavity and amniotic fluid *i.e.* from 57.1 (positive control) to 0 % (treatment group) bacterial growth. Earlier, the *in-vitro* data showed antibacterial nature of G_4-PAMAM-OH at higher concentration and the in-vivo results show that amniotic fluid samples for different fetus in treatment group-3 were found to be negative. The comparison of cytokine expression in placenta of the treatment group, negative and positive control groups shows that the cytokine levels (TNFα and IL-6) in treatment group are comparable to the negative control while they are overly expressed in positive controls (Fig. 8). These results indicate the potential of G_4-PAMAM-OH to effectively kill gram negative bacteria *E. coli* in cervix of guinea pig and prevent chorioamnionitis. This is a significant finding since the chorioamnionitis is known to cause fetal brain injury (Patrick et al., 2004) which could possibly be averted by treatment with G_4-PAMAM-OH as indicated from these findings. It is interesting to note that the G4-PAMAM dendrimers show very negligible transport across the fetal membranes and can therefore be possibly used for selective treatment of mother without crossing into the fetus when administered by intravaginal route (Menjoge et al., 2010).

4. Conclusion

The bactericidal activity of hydroxyl and carboxylic acid terminated PAMAM dendrimer was evaluated against gram negative *E. coli* and compared with amine terminated PAMAM dendrimers. The antimicrobial assay, SEM analysis, cell integrity, inner and outer membrane permeability assays showed that G4-PAMAM-OH and G3.5-PAMAM-COOH dendrimers affect the cell wall of *E. coli*, and were antibacterial at the concentrations evaluated. The major finding was the bactericidal effect of G_4-PAMAM-OH dendrimer and its ability to treat *E. coli* infections *in-vivo* in pregnant guinea pigs. Topical cervical application of 500 μg of G_4-PAMAM-OH treated the *E. coli* infections induced in guinea pig model of chorioamnionitis. The amniotic fluid collected from different fetus in the infected guinea pigs, post treatment showed absence of *E. coli* growth in the cultures plated with it. The cytokines levels were higher in the positive controls confirming presence of infection after inoculation with *E. coli*. The cytokine expression (TNFα and IL-6) in the treatment group was comparable to that in negative control showing the efficacy of G_4-PAMAM-OH to treat the *E. coli* infections. The G4-PAMAM-NH_2 dendrimer is known to be potent antibacterial agent, however, it was found to be highly cytotoxic to above 10 μg/ mL to human cervical epithelial (End1/E6E7) cells and immune cells (BV-2) while the G_4-PAMAM-OH dendrimer was non cytotoxic upto 1mg /mL concentrations to both cell lines. Each dendrimer appears to affect the bacterial cell wall in a different way. The possible mechanisms involve the G_4-PAMAM-NH_2 acting as polycation binding to the polyanionic lipopolysaccharide, the G_4-PAMAM-OH binding via hydrogen bonds to the hydrophilic O-antigens and the $G_{3.5}$-PAMAM-COOH acting as a polyanion chelating the divalent ions in outer cell membrane. The outer and inner membrane permeabilization assay shows that G_4-

PAMAM-OH brings major structural changes to the outer membrane whereas G_4-PAMAM-NH_2 brings major changes to both outer and inner membrane.

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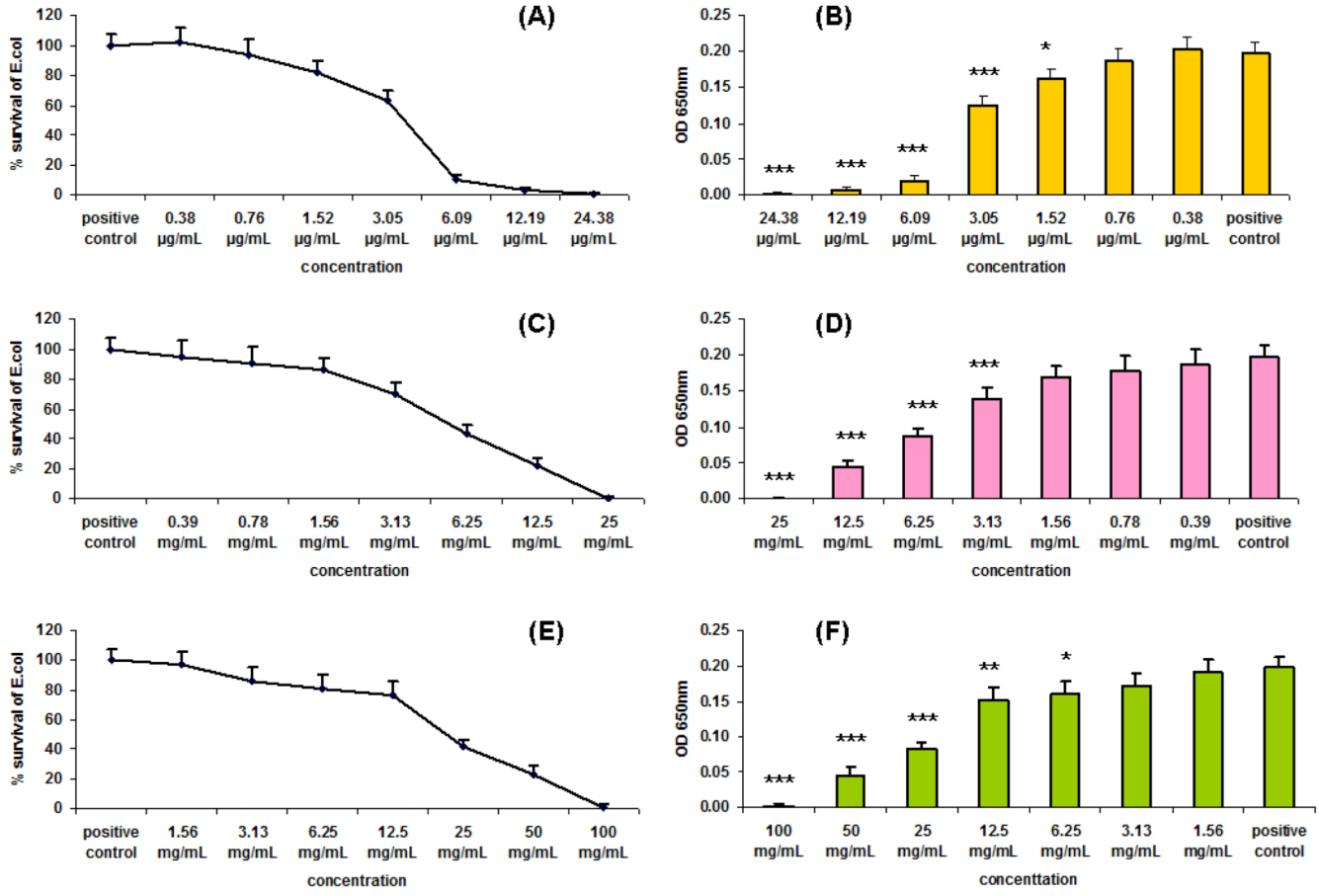


Fig. 1.

Bacterial growth inhibition assays. *E.coli* was treated with the indicated concentration of G_4-PAMAM-NH_2 (**A**) and (**B**), G_4-PAMAM-OH (**C**) and (**D**), $G_{3.5}$-PAMAM-COOH (**E**) and (**F**) dendrimers for 18 h. The initial concentration used for bacterial seeding was 5×10^5 CFU/mL. Three samples were in each group. Bacterial growth was measured by turbidity as the optical density at 650 nm using a microplate reader. * P<0.05, ** P<0.01, *** P<0.001 *VS* Positive control.

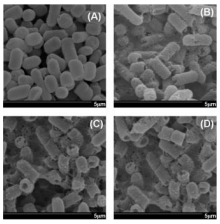


Fig. 2.

SEM images of *E.coli*. (A) untreated *E.coli* (B) 8h treatment of $G_{3.5}$-PAMAM-COOH (C) 8h treatment of G_4-PAMAM-OH (D) 8h treatment of G_4-PAMAM-NH_2. Magnification 20000 ×. Scale bars indicate 5 μm. The treatment with dendrimers shows the damage to the bacterial cell wall.

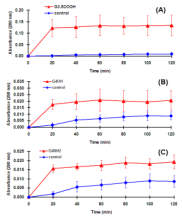


Fig. 3.

Release of intracellular components of *E. coli* suspensions treated with (A) $G_{3.5}$-PAMAM-COOH, (B) G_4-PAMAM-OH and (C) G_4-PAMAM-NH_2. Four samples were evaluated in each group. The increase in the absorbance is an indicator of the compromised cell integrity resulting in leaching of the nuclear components which are absorbed at 260nm.

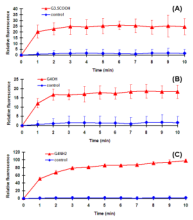


Fig. 4.
Uptake of NPN by *E.coli* suspensions treated with (A) $G_{3.5}$-PAMAM-COOH, (B) G_4-PAMAM-OH and (C) G_4 -PAMAM-NH_2. Four samples were in each group.

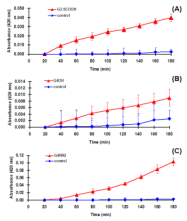


Fig. 5.

Release of cytoplasmic β-galactosidase of *E.coli* treated with (A) $G_{3.5}$-PAMAM-COOH, (B) G_4-PAMAM-OH and (C) G_4-PAMAM-NH_2. Four samples were in each group.

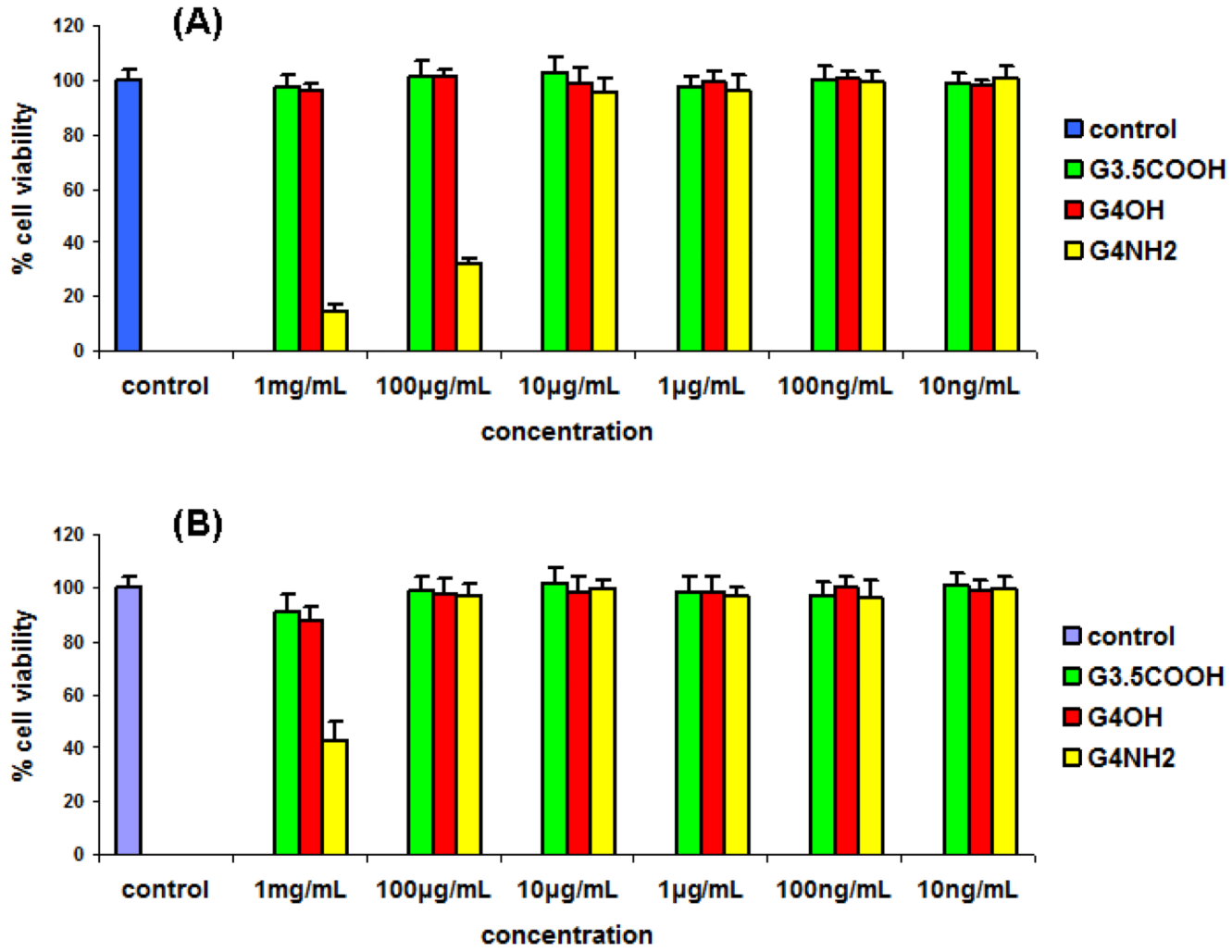
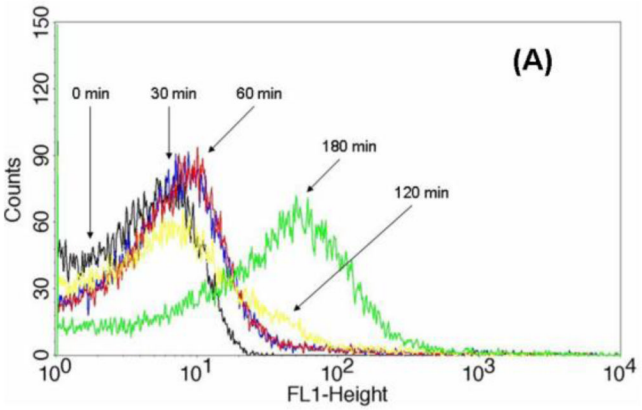
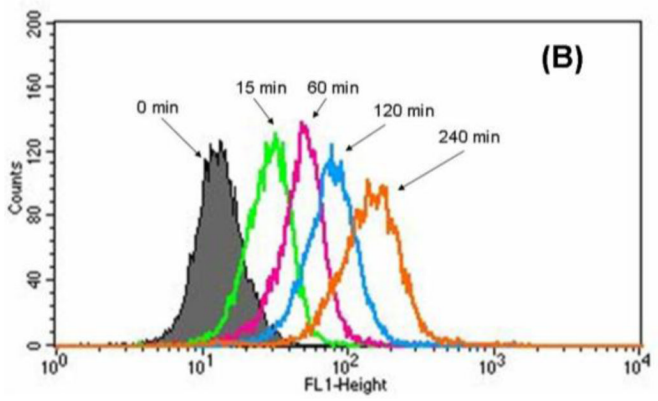


Fig. 6.

Cytotoxicity assay. (A) Human cervical epithelial End1/E6E7 cells and (B) mouse microglial cells were treated with the G_4-PAMAM-OH, G3.5-PAMAM-COOH and G_4-PAMAM-NH_2 dendrimers at concentrations indicated for MIC values. Three samples were in each group. Cell viability was assessed by MTT method. The proportion of viable cells in the treated group was compared to that of negative control.



Flow cytometry of the cell entry dynamics of G4OH-FITC in E.coli



Flow cytometry of the cell entry dynamics of G4OH-FITC in BV-2 cells

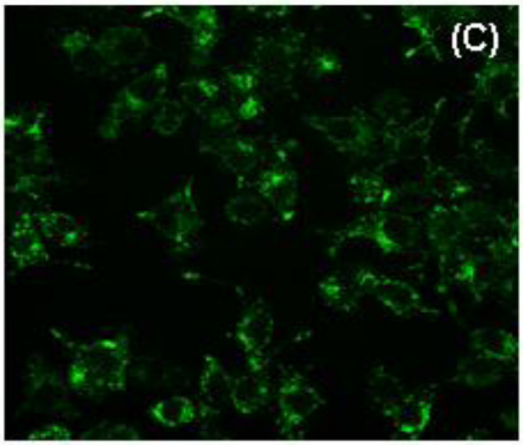


Fig. 7.

Flow cytometry of the cell entry dynamics of (A) G_4-PAMAM-OH-FITC in *E. coli* and (B) BV-2 microglial cell line. The log of FITC absorption intensity (FL1-H on x-axis) is plotted against the number of cells (counts on y-axis). The maximum uptake of G_4-PAMAM-OH-FITC in *E. coli* occurs at 3h. The rapid cellular uptake of G4-PAMAM-OH-FITC within 15 min in microglial cells is evident. The transport of conjugate into microglial cell increased with increasing time. Confocal microscopy images (400×) showed that G_4-PAMAM-OH-FITC appeared to be mainly localized in the cytoplasm of BV-2 cells while the nucleus appeared to be relatively free of the presence of any fluorescence at this time point (C).

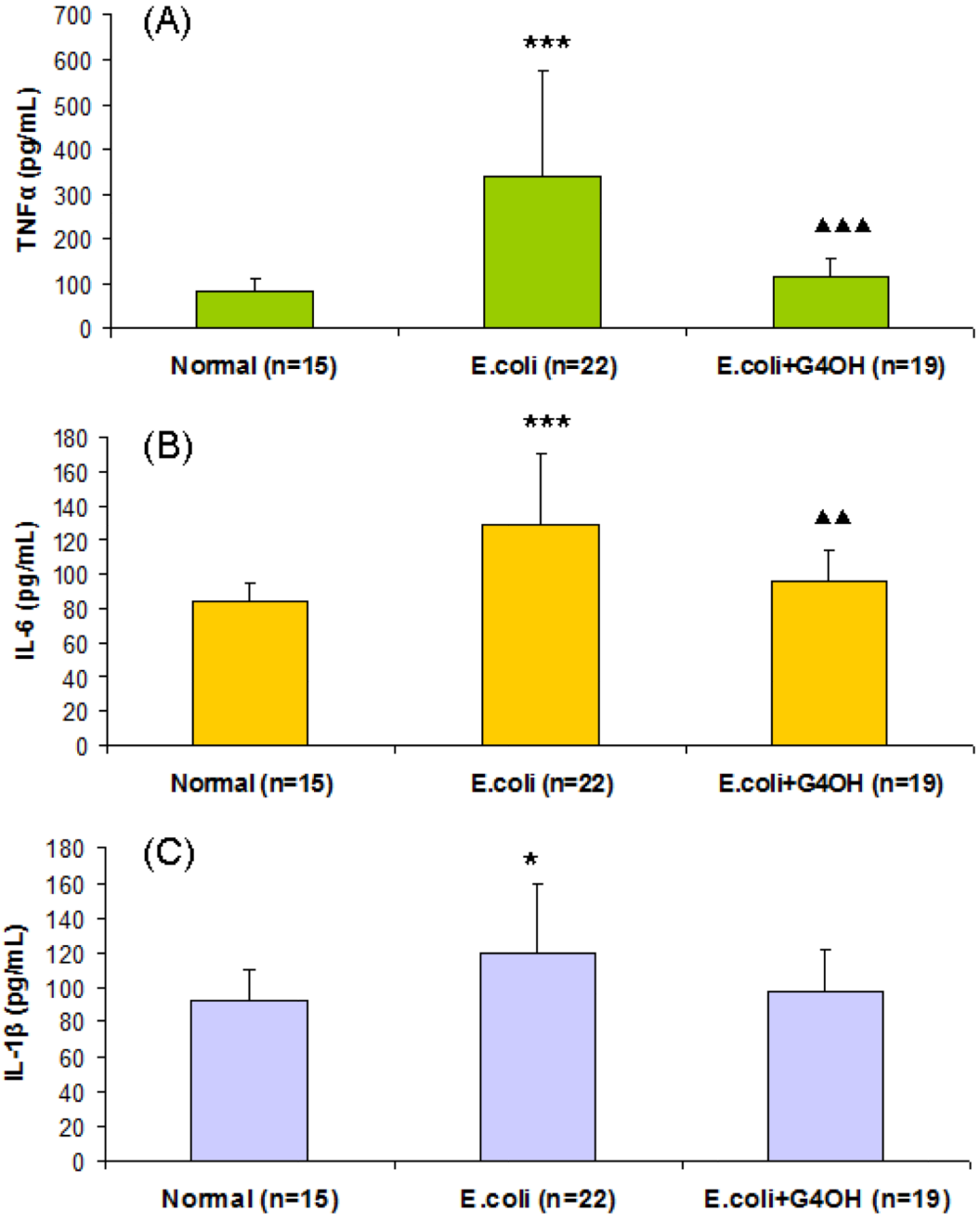


Fig. 8.

The placental tissue (0.3 g) was homogenized in 1 ml RIPA lysis buffer. The homogenate was kept on ice for 30 min and the protein concentration of supernatant was determined. Cytokines concentrations were measured in the total protein fraction using ELISA. (**A**) TNFα measurements in normal, E.coli infected and G4-PAMAM-OH treated guinea pigs (B) IL-6 measurements in normal, E.coli infected and G4-PAMAM-OH treated guinea pigs (C) IL-1β measurements in normal, E.coli infected and G4-PAMAM-OH treated guinea pigs * P<0.05, *** P<0.001 *Vs*. Normal control. ▲▲ P<0.01, ▲▲▲ P<0.001 *Vs. E.coli* group.

Table 1

The inhibition of *E. coli* growth after treatment with G_4-PAMAM-OH dendrimer in guinea pig model of chorioamnionitis

Inoculation with *E.coli*				**Treatment : With G4-OH after *E. coli* inoculation**		
Guinea Pig	**Amniotic fluid from different gestational sacs**			**Guinea Pig**	**Amniotic fluid from different gestational sacs**	
	Bacterial growth	**%**			**Bacterial growth**	**%**
Mother 1	Fetuses (4/5)	80.0		Mother 1	Fetuses (0/5)	0
Mother 2	Fetuses (5/6)	83.3		Mother 2	Fetuses (0/6)	0
Mother 3	Fetuses (1/4)	25.0		Mother 3	Fetuses (0/5)	0
Mother 4	Fetuses (2/5)	40.0		Mother 4	Fetuses (0/3)	0
	Average	57.1			Average	0

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