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In Vitro Evaluation and Targeting of *E. Coli* Internalized by Endothelial Cells using Albumin Microspheres Loaded with Gentamicin

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ABSTRACT The purpose of this study was to evaluate the use of microencapsulated form of gentamicin and the traditional solution form for its intracellular bactericidal effect. Bovine serumalbumin (BSA) microspheres loaded with gentamicin were prepared by using Mini Spray Dryer. Human microvascular endothelial cells (HMECs) were exposed to increasing concentrations of *Escherichia coli* leading to internalization of *E. coli*. The internalized bacteria were targeted using either the microencapsulated or the solution form of gentamicin. The treatment groups using gentamicin solution form and microsphere form showed almost 46% and 86% inhibition in the growth of the internalized bacteria, respectively.

KEYWORDS Gentamicin, Microspheres, Endothelial, Phagocytosis, Cytochalasin D

INTRODUCTION

The clinical response to antimicrobial therapy does not reflect the in vitro results. This is mainly attributed to the inability of the antibacterial agents to penetrate the cell membrane and also is the reason for the failure in the treatment of certain infections caused by the intracellular invasion of the bacteria. Certain microorganisms have the ability to survive and multiply within the cells (Utili et al., 1991) and thus pose a significant challenge in their treatment.

Microorganisms that survive intracellularly may get entrapped in the intracellular space after exit from the endosomal compartment with or without the fusion of the phagosomal vacuole with lysosomes; they may reside in phagosomes, or reside in lysosomes when the endocytic cascade has been completed (Allen & Aderem, 1996; Aderem & Unerhill, 1999).

Phagocytosis is normally considered to be the process in which polymorphonuclear granulocytes, monocytes and macrophages so-called as professional phagocytes are involved. This distinction between professional and nonprofessional phagocytes arose some 25 years ago when RBCs were found to be taken up by macrophages and mouse fibroblasts, the main difference between the two lies in the range of particles the two can engulf, this is mainly because of the lack of efficient

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phagocytic receptors such as those for immunoglobulin and complement displayed by macrophages and polymorphonuclear granulocytes. Paraprofessional cells are another set of specialized cells such as dendritic cells that indulge in selective phagocytic activities. They take up certain particles as well as microorganisms such as mycobacteria. Retinal epithelial cells are known to nibble the effete tips of retinal rods and cones (Rabinovitch, 1995).

Earlier experiments in our lab have shown that endothelial cells although being nonprofessional phagocytic cells do phagocytose microspheres; however, the role of endothelial cells in internalizing infectious organisms such as *Escherichia coli* is yet to be evaluated. These cells play a very important role in the pathogenesis of sepsis (Aird, 2001). It is known that various stimuli such as release of lipopolysaccharide from the bacterial cell wall can cause release of large amount of cytokines such as IL-1, IL-6, and TNF-α, which in turn can contribute to the microvascular dysfunction, impaired tissue oxygenation and organ injury.

Biodegradable microspheres especially those using albumin as a polymer matrix have received tremendous attention over past few decades (Okada & Toguchi, 1995) and have been widely used in encapsulating therapeutic agents such as azidothymidine, vaccine adjuvants, and antibodies. A recent study done in our laboratory on *E. coli* induced peritonitis model using albumin microspheres containing neutralizing antibodies to TNF-α, IL-1β either alone or in combination with gentamicin solution showed significant improvement in the survival even with a delay in the administration of the microspheres (Oettinger et al., 1999a,b; Oettinger et al., 2003). Hence, a microencapsulated form of gentamicin certainly warrants an investigation in the sepsis condition especially against an infection caused by *E. coli* bacteria.

The purpose of this present study is to evaluate the use of gentamicin in both the solution and the microparticulate forms in the intracellular targeting of *E. coli* internalized by the human microvascular endothelial cells (HMECs).

The traditional solution form of the drug used in treatment of sepsis kills the infectious organism in the flowing blood, but is not able to effectively penetrate the cells lining the blood vessels where the bacteria must have been internalized. Microparticles have the ability to penetrate the cells more effectively and also sustain the release of drugs over a period of time, which helps in reducing the dosing frequency and the toxicity of the drug (Oettinger et al., 1999a,b).

MATERIALS AND METHODS

E. coli Cell Culture

E. coli (ATCC # 8739) was obtained from ATCC (Rockville, MD) and grown until midlog phase at 37°C and stored at 4°C until ready to use. Trypticase soy agar (TSA), trypticase soy broth, concentrated sulfuric acid, barium chloride and petri plates were obtained from Fischer Scientific, Norcross, GA. Syto 9 labeling kit was obtained from molecular probes, Eugene, Oregon.

HMECs Culture

Cytochalasin D was obtained from Sigma Chemical Company, St Louis, MO. HMECs and the nutrient media [MCDB, supplemented (EBM)] were generously gifted by Centers for Disease Control and Prevention, Atlanta. HMECs were grown in 75 cm^2 flasks and then subcultured in 24-well tissue culture plates and incubated at 37°C and 5% CO_2 for 24 hr (80–90% confluent).

Microsphere Preparation

Butanol and glutaraldehyde were obtained from Fischer Scientific, Norcross, GA. Buchi B-191 Mini Spray Dryer purchased from Brinkmann instruments, available in-house was used for preparation of microspheres.

A homogenous mixture of gentamicin:bovine serum albumin (BSA) (1:5) dissolved in deionized water so as to make a 5% solution was prepared and subjected to spray drying. Spray drying was performed using a Mini Spray Dryer (Buchi B-191). Using compressed air from an in-house supply (700 NL/hr), a fluid nozzle atomized the homogenous solution at a pump setting of 4%, the filtered air was aspirated at 95%, the inlet temperature was set at 110°C and operation under these standard conditions gave an outlet temperature of 73°C. The solvent evaporation by the flow of heated air formed the solid microparticles, which separated in a cyclone and settled down in the collector.

Microsphere Cross-linking

The microspheres obtained were further stabilized using varying concentrations of glutaraldehyde as a cross-linking agent. A total of 200 mg of albumin microspheres were suspended in 50 mL of butanol. Glutaraldehyde was added to this suspension in order to achieve a

final concentration of 0.4%, 2%, and 4% (v/v). The cross-linking was performed for 1 hr, after which the microsphere suspension was filtered off and the microspheres obtained were then dried at 37°C overnight in order to remove any trace amounts of butanol and glutaraldehyde.

F1 = Formulation prepared as described above for preparation of microspheres with 0.4% glutaraldehyde concentration.

F2 = Formulation prepared as described above for preparation of microspheres with 2% glutaraldehyde concentration.

F3 = Formulation prepared as described above for preparation of microspheres with 4% glutaraldehyde concentration.

Gentamicin Encapsulation & Particle Size Analysis

Ten milligrams of microspheres (F1, F2, and F3) were weighed and crushed in pestle and mortar and 10 mL of PBS (pH 7.4) was added to it. Of this mixture, 5 mL was then placed in a dialysis bag and the bag was suspended in 40 mL of PBS (pH 7.4) and the drug sample from outside the dialysis bag (MWCO:6–8 kDa.) was analyzed after 2 hr on a Perkin-Elmer Lamba 4B UV/visible spectrophotometer at a wavelength of 256 nm. The particle size analysis was done using a laser particle counter. Microspheres were suspended in 10 mL deionized water filtered through 0.2 μm and the particle size was measured using spectrex laser particle counter (Spectrex Laser Particle Counter PC 2000, Redwood City, CA).

In Vitro Release Studies

An in vitro release study was performed by placing weighed amount of gentamicin microspheres in a dialysis bag (MWCO 6000–8000, spectrum labs) containing 5 mL of phosphate buffer solution (pH 7.4) and suspended in 35 mL of the same buffer solution. The temperature was maintained at 37°C. At predetermined intervals, a 3 mL sample was withdrawn, analyzed and replaced with fresh buffer. The content was analyzed using a UV-Vis spectrophotometer at a wavelength of 257 nm. In vitro release studies were also performed in 0.125% trypsin to mimic intracellular conditions. Microspheres were weighed and suspended in a dialysis bag containing 0.125% trypsin. The dialysis bag was finally suspended in phosphate buffered saline (PBS) in a beaker and the samples were later analyzed at different time points from the beaker. The control in both the release studies was un-cross-linked microspheres.

Uptake of Labeled Albumin Microspheres Loaded With Gentamicin by the Endothelial Cells

BSA microspheres (cross-linked with 4% glutalraldeyde) loaded with gentamicin were labeled with fluorescamine, and exposed to HMECs. At different time points, the cells were washed with PBS (pH = 7.4) so as to remove the extracellular microspheres and observed under a fluorescent microscope

Internalization of *E. coli* by the Endothelial Cells

In order to evaluate the internalization of bacterial organism by the endothelial cells, the bacterial organism *E. coli* was stained with the fluorescent dye, exposed to HMECs and the microscopic images were obtained to determine its uptake (qualitative study). Also, to enumerate the total number of bacteria engulfed by the HMECs, these cells were exposed to *E. coli* and were lysed at various time points and the engulfed bacteria were grown on trypticase soy agar plate to determine its rate of uptake (quantitative study).

Qualitative Study

HMECs were grown on tissue culture plates. *E. coli* was labeled with Syto 9 stain obtained from molecular probes and exposed to HMECs at different concentrations (1:1 to 1:1000). At different time points, the HMECs were washed with PBS and gentamicin (100 μg/mL) and incubated for 1 hr in order to remove the extracellular *E. coli* and cells were observed under the fluorescent microscope.

Quantitative Study

HMECs were grown on tissue culture plates until they were 80–90% confluent. *E. coli* bacteria were then exposed to HMECs at different concentrations (1:1 to 1:1000). At different time points, the HMECs were washed with PBS and gentamicin (100 μg/mL) and incubated for 1 hr in order to remove the extracellular *E. coli.*

Following internalization of bacteria the HMECs were lysed using Triton-X (1%) and the number of

E. coli internalized were enumerated by plating the lysed media on trypticase soy agar plate, which were incubated overnight and the number of individual colonies on the agar plate were recorded.

E. coli uptake inhibition Studies using cytochalasin D

In order to determine if phagocytosis process was the mechanism behind uptake of the bacterial organism by the HMECs, the cells were treated with cytochalasin D (a potent phagocytic inhibitor) prior to *E. coli* being exposed to HMECs and the uptake mechanism was evaluated both qualitatively as well as quantitatively.

Qualitative Study

HMECs were grown on tissue culture plates. These cells were later on exposed to the drug cytochalasin D following which they were incubated for 1 hr and the cells were then washed off with PBS (1X) and exposed to *E. coli* labeled with Syto 9. Following the uptake of bacteria, the cells were washed with PBS and gentamicin (100 μg/mL) and incubated for 1 hr in order to remove the extracellular *E. coli* and cells were observed under the fluorescent microscope.

Quantitative Study

HMECs were grown in 24-well tissue culture plates until they were 80–90% confluent, these cells were then exposed to the drug cytochalasin D, incubated for 1 hr and the cells were then washed off with PBS (1X) and exposed to *E. coli*. Following the uptake of bacteria, the cells were washed with PBS and gentamicin (100 μg/mL) and incubated for 1 hr in order to remove the extracellular *E. coli*. The HMECs were later on lysed using Triton-X (1%) and the number of *E. coli* internalized were enumerated by plating the lysed media on trypticase soy agar plate, which were incubated overnight and the number of individual colonies on the agar plate were recorded.

In Vitro Targeting of *E. coli* Internalized Within the Endothelial Cells

Either albumin microspheres loaded with gentamicin (cross-linked with 4% glutaraldehyde) in the suspension form (16 μg/mL calculated based on the drug loading of 1:5), gentamicin solution (16 μg/mL) or HMECs nutrient media (control group) was exposed to HMECs containing *E. coli* in three different groups of treatment.

Simultaneous (Immediate) Treatment

In this group, the HMECs were treated with the formulations immediate to the phagocytosis of *E. coli*, and the HMECs were then lysed at different time points using Triton-X (1%) and allowed to stand for 1 hr at 4°C. The total number of *E. coli* present at each time point was evaluated for the solution, microsphere and control group.

Prophylactic Treatment

In this group, the HMECs were grown to 90% confluency on a 24-well tissue culture plate and once confluent were either treated with gentamicin solution, microspheres, or HMECs nutrient media (control group) for 2 hr, following which the cells were washed with PBS (pH = 7.4) and exposed to *E. coli* for 4 hr because 4 hr was the time needed for the cells to phagocytose the bacteria. The HMECs were then washed with PBS (pH = 7.4) and gentamicin (100 μg/mL) in order to remove the extracellular bacteria.

The HMECs were then lysed using Triton-X (1%) at different time points over 24 hr and allowed to stand for 1 hr at 4°C. The total number of bacteria present postlysis was evaluated for the solution, microsphere formulation, and the control group.

Delayed Treatment

The formulations or the nutrient media were exposed to the HMECs 8-hr postexposure of *E. coli*. The HMECs were then lysed at various time points using Triton-X (1%) and allowed to stand for 1 hr at 4°C .The total number of *E. coli* present at each time point was evaluated postlysis for the solution, microsphere formulation and the control group.

Statistical Analysis

Data were analysed using Student's *t*-test or repeated measures ANOVA as appropriate.

RESULTS

In order to determine the total amount of drug encapsulated, the content of gentamicin in 10 mg of microspheres was determined and the actual content was found to be 15.83%, whereas the theoretical loading was 16.66% (based on 1:5 ratio, 1 = gentamicin and 5 = BSA) giving us an encapsulation efficiency of 95%.

The particle size obtained was in the range of 2–7 μm, the average particle size of the microsphere showed a slight increase (Table 1) with the increasing amount of glutaraldehyde concentration.

In Vitro Release Studies

The release kinetics for the gentamicin microspheres demonstrated biphasic pattern of drug release, because of the drug being present on the surface of the microspheres and remaining being encapsulated in the core. The un-cross-linked microspheres showed approximately 100% drug release within 2 hr whereas the 4% cross-linked microspheres showed 56% and 58% drug release in PBS and Trypsin, respectively in 2 hr (Table 2).

Uptake of Labeled Albumin Microspheres Loaded With Gentamicin by the Endothelial Cells

The uptake studies indicate that the endothelial cells have the ability to phagocytose albumin microspheres and this phagocytic uptake increases with the increase in time between 1 and 4 hr of exposure of the gentamicin microspheres to the endothelial cells. (Data published elsewhere)

Internalization of *E. coli* Bacteria

Qualitative Study

The HMECs show an increase in uptake of *E. coli* with the increase in dose and time, as seen in the fluorescent images (Fig. 1) taken using the fluorescent microscope, the images show the internalization of the bacteria within 4 hr, with most of the bacteria present in the cytoplasm of the endothelial cells, also the images taken at 24 hr time (Fig. 2) show an increase in the uptake of bacteria with most of the HMECs being saturated by *E. coli*.

Quantitative Study

E. coli bacteria were enumerated by determining the absorbance at 680 nm. A McFarland standard curve was made by serially diluting the stock solution of barium chloride (1 mg/mL) in deionized water. The absorbance of stock solution corresponded to the bacterial count of 1×10^8 cfu/mL. The HMECs showed an increase in the uptake of *E. coli* with time and with the increase in the amount of *E. coli* exposed to the endothelial cells, thus confirming the phagocytic ability of the endothelial cells. The highest concentration used was 1:1000 which showed a decrease in the uptake of the bacteria, suggesting death of endothelial cells caused by a significant increase in the bacterial biomass (Fig. 3).

TABLE 1 **Influence of Glutaraldehyde Concentration on Particle Size of Microspheres**

Formulation	Percent glutaraldehyde used	Mean particle size (μm)	Standard Deviation
F1	0.4%	4.21μm	2.91
F2	2%	4.20μm	2.76
F3	4%	5.73μm	2.69

TABLE 2 **Comparison of the Amount of Drug Released From Albumin Microspheres Loaded With Gentamicin in PBS and Trypsin at 2 hr**

Media	Un-cross-linked microspheres	0.4% cross-linked microspheres	2% cross-linked microspheres	4% cross-linked microspheres
PBS(pH = 7.4)	99%	88%	64%	56%
Trypsin (0.125%)	100%	78%	76%	58%

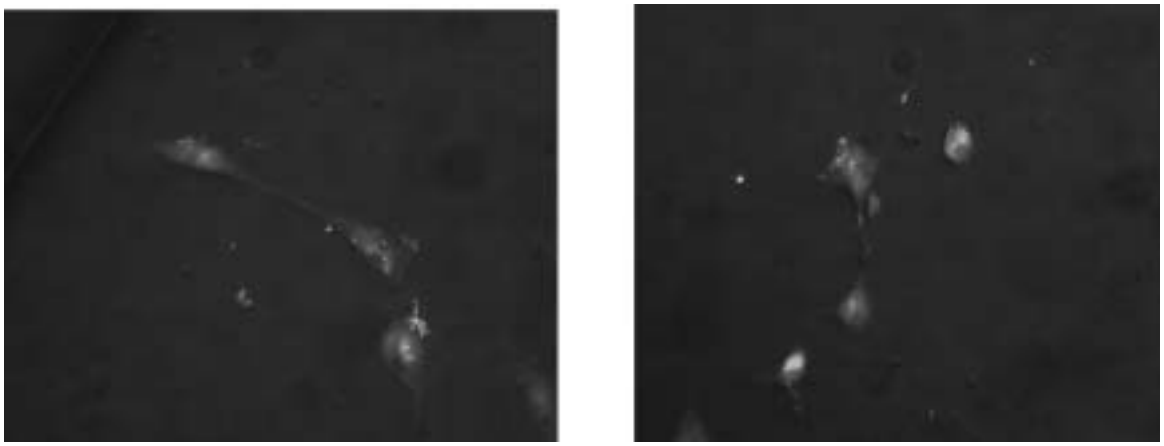


FIGURE 1 **Phagocytic Uptake of *E. coli* by Endothelial Cells Taken 4 hr Postexposure of *E. coli* to Endothelial Cells Using Fluorescent Microscope (Magnification 50×).**

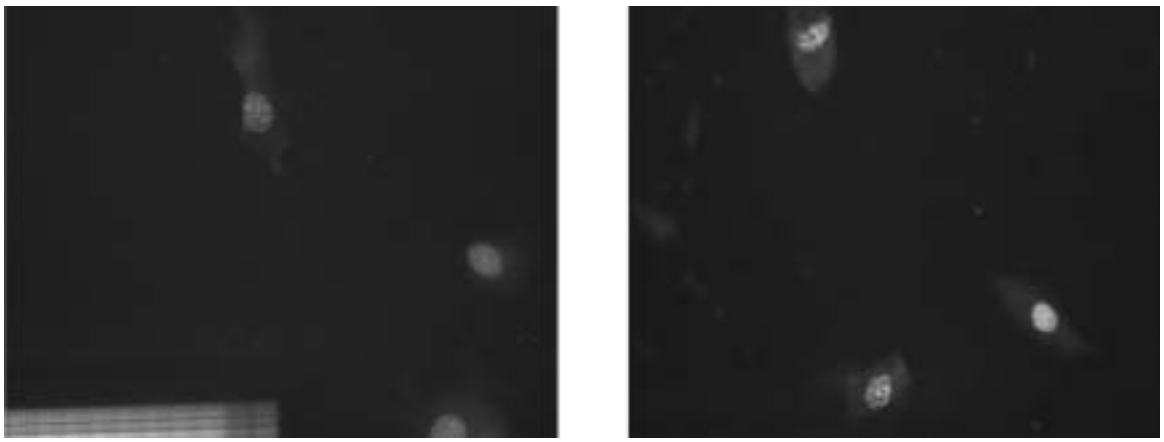


FIGURE 2 **Phagocytic Uptake of *E. coli* by Endothelial Cells Taken 24 hr Postexposure of *E. coli* to Endothelial Cells Using Fluorescent Microscope (Magnification 50×).**

Uptake Inhibition by Cytochalasin D

Cytochalasin D when preexposed to the HMECs significantly decreased the uptake efficiency of the endothelial cells. The uptake of *E. coli* increased with the increasing bacterial concentration in the cells not treated with cytochalasin D, but the uptake was lower with the HMECs pretreated with cytochalasin D (Figs. 4 and 5).

Targeting of *E. coli* Internalized Within the Endothelial Cells

Simultaneous Treatment

Compared to the control group, the treatment of HMECs infected with *E. coli* showed 86% and 46% inhibition in growth and uptake of *E. coli* using gentamicin microspheres and gentamicin solution, respectively (Fig. 6).

Prophylactic Treatment

Compared to the control group, the prophylactic treatment of HMECs on uptake of bacteria showed 78% inhibition in growth of *E. coli* with gentamicin microspheres and 10% inhibition in the growth with gentamicin solution (Fig. 7).

Delayed Treatment

Compared to the control group, the delayed treatment of HMECs on uptake of bacteria showed 80% inhibition in the growth and uptake of *E. coli* with gentamicin microspheres and 50% inhibition with gentamicin solution (Fig. 8).

The solution form of gentamicin does not permeate the endothelial cells as effectively and as a result does not show significant inhibition in the bacterial uptake and growth, however, the microspheres as shown in

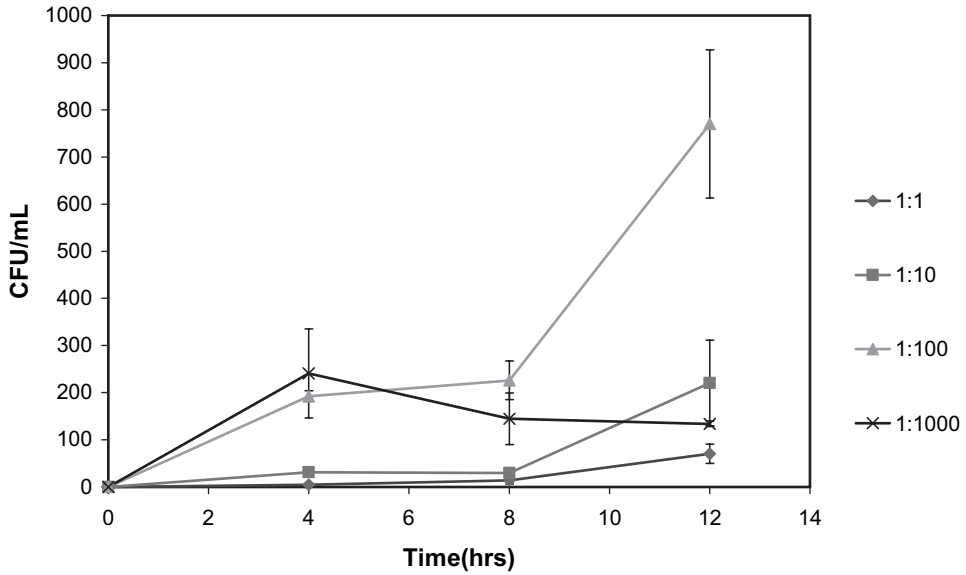
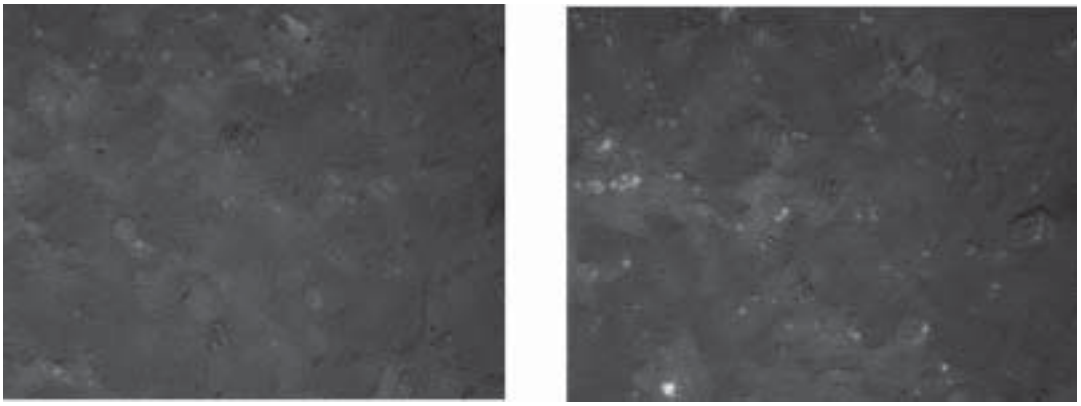


FIGURE 3 Uptake of *E. coli* in HMECs. (1:1), (1:10), (1:100), (1:1000) Represents the Ratio of Endothelial Cells to *E. coli*. Values are Means +/– SE From Six Replicates. CFU/mL = Colony Forming Unit Present per mL of the Sample (Represents the Number of Colonies Formed on the Trypticase Soy Agar Plate).



(a) ***E.Coli*** **uptake by the endothelial cells at 3hrs with Cytochalasin D**

(b) ***E.Coli*** **uptake by the endothelial cells at 3 hrs without Cytochalasin D**

FIGURE 4 Effect of Phagocytosis Inhibitor Cytochalasin D on *E. coli* Uptake by the Endothelial Cells.

the earlier studies can be phagocytosed by the endothelial cells and thus show a higher inhibition in the bacterial invasion compared to the solution formulation.

DISCUSSION

Albumin is used as a polymer matrix since it is a biodegradable and biocompatible polymer; moreover, it has been used in past several decades to obtain microspheres loaded with a large variety of drugs. As shown in Table 1, microspheres of desirable size were obtained for the phagocytosis process by the endothelial cells, it also shows that spray drying is a convenient, safe, and a reproducible method of obtaining smaller microparticles (Palmieri et al., 2001) which can be easily scaled up to produce a larger batch size. Glutaraldehyde as a cross-linker can be used in different concentrations to tailor the release of drug from the albumin microspheres thus offering a controlled delivery of the drug (Anthony, 1985). These microspheres can also be effectively labeled using a fluorescent stain such as fluoroscamine and also can be phagocytosed by the endothelial cells.

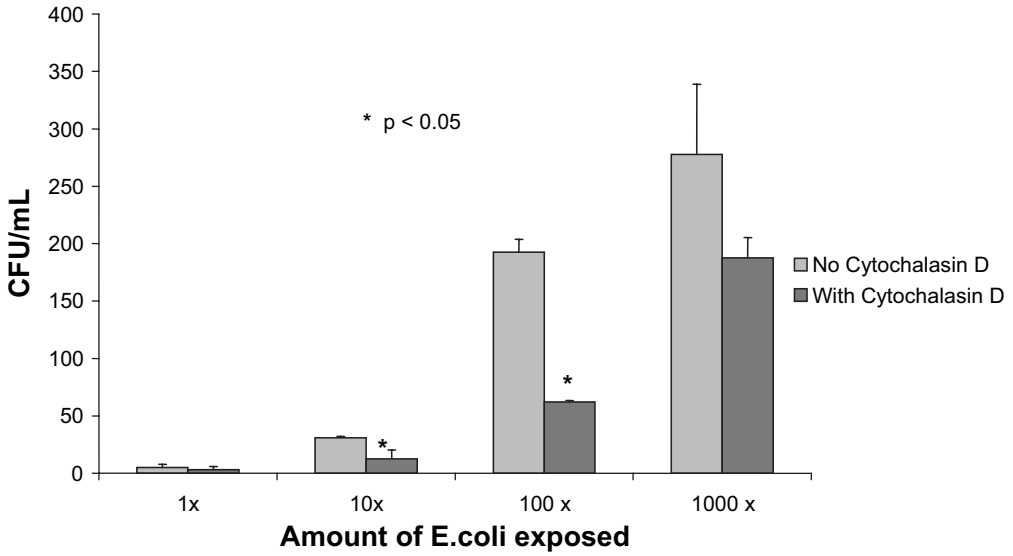


FIGURE 5 **Effect of the Phagocytosis Inhibitor Cytochalasin D on Uptake of *E. coli* by the Endothelial Cells. The Uptake Increased With the Increasing Bacterial Concentration in Both the Groups but the Uptake was Significantly Lower in the Group of Endothelial Cells Preexposed to Cytochalasin D. Values are Means +/– SE From Six Replicates. *$p < 0.05$ Compared With no Cytochalasin D.**

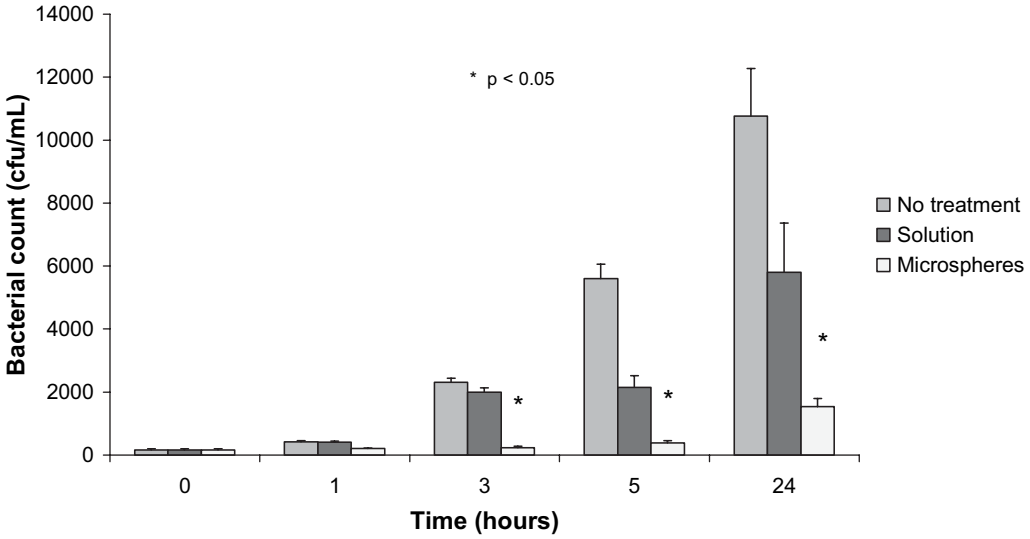


FIGURE 6 **Simultaneous Treatment of HMECs Infected With *E. coli* Using Gentamicin Microspheres and the Solution Formulation. Values are Means +/– SE From Six Replicates. *$p < 0.05$ Compared With no Treatment and Solution.**

Internalization and survival of bacteria such as *Staphylococcus aureus* by the endothelial cells has been studied in the past (Menzies et al., 1998; Hess et al., 2003), which indicates phagocytosis as the possible mechanism of uptake, thus making endothelial cells as an important target site to counteract the infection. This study, however, is the first study to demonstrate the endothelial cells as the target cells for invasion by *E. coli*. Syto 9 was effectively used to label the live *E. coli* bacteria thereby enabling us to track the internalization of *E. coli* within the HMECs as confirmed by fluorescent microscopy. This also confirms that endothelial cells do play a role in phagocytosis process, which could be a key site in effectively treating the infection caused by different pathogens leading to septic shock condition and thus help in preventing the organ damage caused as a result of sepsis.

Cytochalasin D is a potent phagocytic inhibitor, which acts by inhibiting the polymerization of the microfilaments present on the surface of endothe-

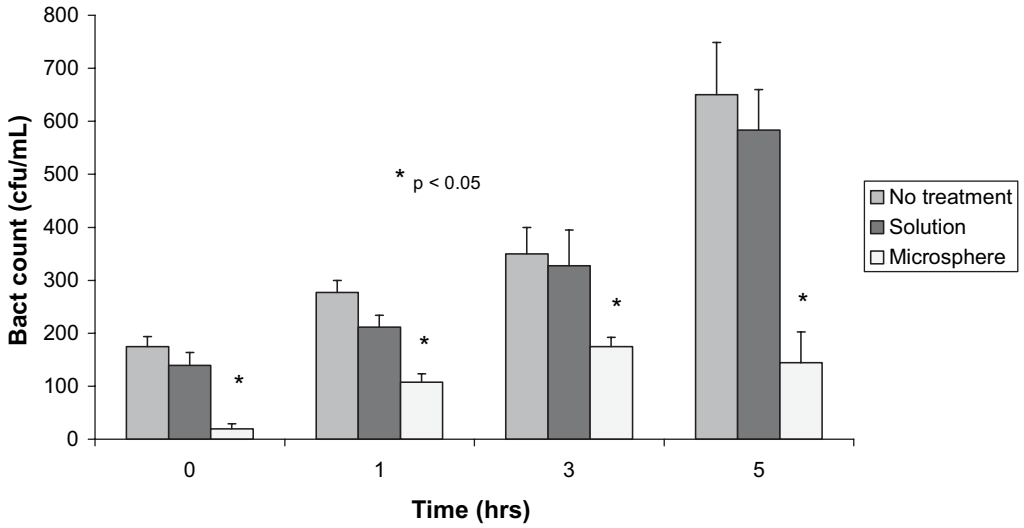


FIGURE 7 **Prophylactic Treatment of HMECs Infected With *E. coli* Using Gentamicin Microspheres and the Solution Formulation. Values are Means +/– SE From Six Replicates. *$p < 0.05$ Compared With no Treatment and Solution.**

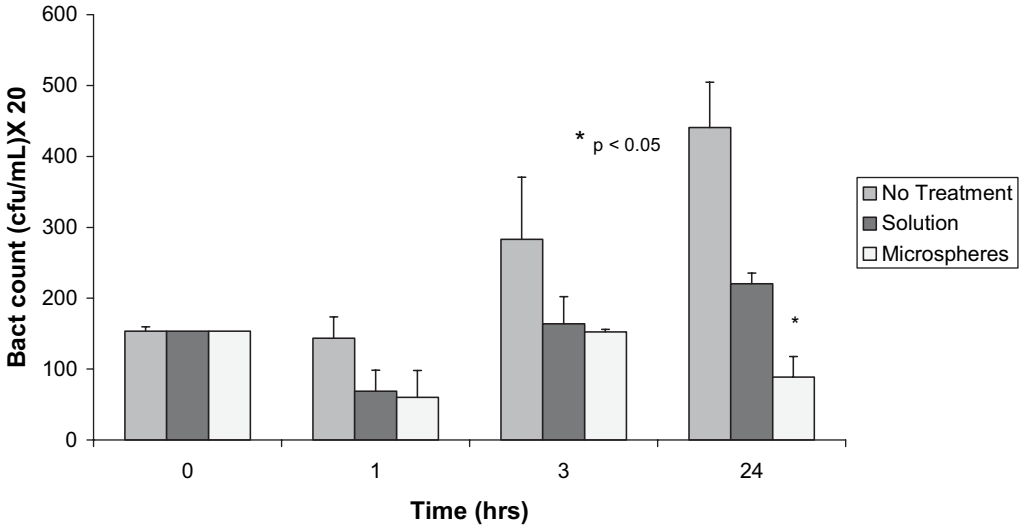


FIGURE 8 **Delayed Treatment of HMECs Infected With *E. coli* Using Gentamicin Microspheres and the Solution Formulation. Values are Means +/– SE From Six Replicates. *$p < 0.05$ Compared With no Treatment and Solution.**

lial cells, thereby decreasing the phagocytosis (Menzies et al., 1998). When HMECs were pretreated with cytochalasin D for 1 hr prior to being exposed to either bacteria or microspheres, it was seen that there was a significant inhibition in the uptake of the *E. coli* by the endothelial cells, thus clearly indicating that phagocytosis is the key mechanism behind the internalization of the bacteria. All the three treatment groups studied here showed consistent results in lowering the bacterial count which was significantly higher with the microsphere group compared to the gentamicin solution or the untreated group.

CONCLUSION

The spray drying method produces porous microspheres which were further stabilized by the use of glutaraldehyde as a cross-linking agent. Also the amount of drug released from the microspheres was controlled by the amount of cross-linking agent used to stabilize the microspheres. The treatment of infected HMECs

with gentamicin solution and microspheres, show that these endothelial cells can be effectively targeted with the use of solution and microspheres, but the effect is more enhanced when gentamicin is used in the microsphere form. This is because, in the phagocytosis process, the drug can be targeted to the endothelial cells compared to the solution form of gentamicin which cannot permeate the endothelial cells as effectively as the microsphere form. Having evaluated the effect of gentamicin microspheres in vitro, it would be interesting to see the effect of these microspheres in a septic shock rat model infected with gram negative bacteria such as *E. coli*.

ABBREVIATIONS

E. coli – *Escherichia coli*, HMECs – Human microvascular endothelial cells, cfu/mL – colony forming unit/milliliter, mg/mL – milligram/milliliter, μm – micrometer, PBS – phosphate buffered saline, μg/mL – microgram/milliliter, TNF-α – tumor necrosis factor-alpha, IL-1β – interleukin-1 beta.

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REFERENCES

Aderem, A., & Unerhill, D. M. (1999). Mechanisms of phagocytosis in macrophages. *Ann. Rev. Immunol*., *17*, 593–623.

Aird, W. C. (2001). Vascular bed-specific hemostasis: Role of endothelium in sepsis pathogenesis. *Crit. Care Med*., *29*, (suppl) S99–S106.

Allen, L. A., & Aderem, A. (1996). Mechanism of phagocytosis. *Curr. Opin. Immunol*., *8*, 36–40.

Anthony, F. Y. (1985). Albumin microsphere: heat and chemical stabilization. *Meth. Enzymol*., *112*, 3–18.

Hess, D. J., Stanley, H. M., Erickson, B. A., Wells, C. L. (2003). Intracellular survival of Staphylococcus aureus within cultured enterocytes. *J. Surg. Res*., *114*(1), 42–49.

Menzies, B. E., Kourteva, I. (1998). Internalization of *Staphylococcus aureus* by endothelial cells induces apoptosis. *Infect. Immun*. *66*(12), 5994–5998.

Oettinger, C., D'Souza, M. J., & Milton, G. (1999a). Evaluation of microspheres containing cytokines neutralizing antibodies in endotoxemia. *Drug Dev. Ind. Pharm*., *25*(6), 727–734.

Oettinger, C., D'Souza, M. J., & Milton, G. (1999b). Targeting macrophages with microspheres containing cytokine-neutralizing antibodies prevent lethality in gram-negative peritonitis. *J. Interferon Cytokine Res*., *19*, 1125–1133.

Oettinger, C., & D'Souza, M. J. (2003). Microencapsulation of tumor necrosis factor oligomers: A new approach to proinflammatory cytokine inhibition. *J. Interferon Cytokine Res*., *23*, 533–543.

Okada, H., & Toguchi, H. (1995). Biodegradable microspheres in drug delivery. *Crit. Rev. Ther. Drug Carrier Syst*., *12*(1), 1–99.

Palmieri, G. F., Bonacucina, G., Di Martino, P., & Martelli, S. (2001). Spray-drying as a method for microparticulate controlled release systems preparation: advantages and limits. I. Water-soluble drugs. *Drug Dev. Ind. Pharm*., *27*(3), 195–204.

Rabinovitch, M. (1995). Professional and non-professional phagocytes: an introduction. *Trends Cell Biol*., *5*, 85–87.

Utili, R., Luigi, E. A., Adinolfi, M. D., Tripodi, M. F., Marrone, A., & Ruggiero, G. (1991). Activity of aminoglycosides against phagocytosed bacteria. *J. Antimicrob. Chemother*., *28*(6), 897–904.