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Antibacterial activity of triclosan chitosan coated graft on hernia graft infection model

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ABSTRACT

The use of mesh in hernia repair has become common, because of lower recurrence rate and simple application. Data from the meta-analysis and the multi-central studies support the use of meshes in hernia repair. One of the complications due to the hernia repair with mesh is the infection. The incidence range is between 1 and 10%. Triclosan embedded commercial absorbable suture materials are used to reduce surgical site infection rate. This study was planned on mesh infection model, because of the low incidence rate. The agent isolated from mesh infections was mostly *Staphylococcus aureus* and thus it was used as the infecting agent in this research. To achieve a better therapeutic efficacy, triclosan was formulated in chitosan gels. Chitosan is an attractive biopolymer because of its biocompatible, biodegradable, bioadhesive properties. Gel formulations using chitosans (low, medium and high molecular weight) were prepared in 1% (v/v) acetic acid solution and in vitro release profiles were evaluated. Gel formulations showed release profile extended up to 7 days and high molecular weight chitosan gel formulation was released higher quantity drug than other formulations. Meshes coated with triclosan loaded chitosan gel were used to reduce bacterial count and to prevent mesh infection in the study. 24 h and simultaneous bacteria inoculation was used to model mesh infection. The rats were observed for 8 days by means of surgical site infection. On the eighth day, the animals were sacrificed and the grafts were removed. Tissue squeezers were used to liberate bacterias from removed grafts. The isolated suspensions were cultured on blood agar plates and colony-forming units were counted overnight. Grafts coated with triclosan loaded chitosan gel presented satisfactory preventive effect against graft infection.

1. Introduction

Chitosan, a natural polymer obtained by alkaline deacetylation of chitin, is a biocompatible and biosorbable biopolymer. These properties make chitosan a good candidate for the development of conventional and novel drug delivery systems. Chitosan is reported to be used as a support material for gene delivery, cell culture, and tissue engineering. It behaves as a hemostatic agent presenting antithrombogenic properties (Rao and Sharma, 1997) and stimulates the immune system of the host against viral and bacterial infections (Tarsi et al., 1997; Je and Kim, 2006). Moreover, chitosan is easily hydrolyzed and metabolized by various chitosanases and lysozyme and considered as biodegradable (Prabaharan et al., 2007). Chitosan, α (1–4) 2-amino-2-deoxy-β-D glucan, has structural characteristics similar to glycosaminoglycans. Chitosan has interesting biopharmaceutical characteristics such as pH sensitivity, biocompatibility and low toxicity (Prabaharan and Mano, 2006; Van et al., 2006). Chitosan always contains a number of N-acetyl-D-glucosamine units and the proportion of these units with respect to the total number of units (N-acetyl-D-glucosamine units plus D-glucosamine units) is represented as the "degree of acetylation" (DA). It is possible to change the deacetylation degree of chitin by changing the conditions of deacetylation reaction, in order to prepare various chitosans which have different DA. In the case of chitosan, DA is considered to be below 50% a structural parameter influencing the solubility, crystallinity, charge density and enzymatic degradation rate of chitosan (Lim et al., 2008). Additionally, cell adhesion and proliferation of certain cell types like keratinocytes, fibroblasts and rat bone marrow stromal cells are reported to be influenced by the DA. Due to biocompatible and biodegradable properties, the use of chitosan and its derivatives as excipients in drug delivery, have been increased in recent years (Khor and Lim, 2001).

Triclosan (2,4,4-trichloro, 2-hydroxydiphenylether) is a non-cationic antimicrobial agent and a lipid-soluble compound. Antimicrobial activity of triclosan against various microbial

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pathogens was evaluated by in vitro studies. It has activity against many, but not all, types of Gram-positive and Gram-negative bacteria, some fungi, *Plasmodium falciparum* and *Toxoplasma gondii* for more than 30 years. It has proven bacteriostatic properties against strains of *Escherichia coli*, *Pinguicula vulgaris*, and *Staphylococcus aureus*, which are resistant to penicillin, streptomycin, and other broad spectrum antibiotics (Jones et al., 2000; Bhargava and Leonard, 1996; Rao et al., 2003). It is bacteriostatic at low concentrations, but at higher concentrations is bactericidal. Triclosan is known to penetrate skin and mucous membranes. Thus, it is used clinically and in oral hygiene products, also is incorporated into many types of cosmetic formulations. It has also been employed as surgical scrubs, handwashes, body washes and in dental hygiene products (Steinberg et al., 2006; Segundo et al., 2005).

Today, surgeons are performing a variety of techniques to repair hernias like the conventional method, tension-free mesh technique, the laparoscopic method. In this study, we used tension-free mesh technique. For this technique, an incision is made at the site of the hernia and a piece of mesh is inserted to the abdominal wall. Recovery is very quick and hernia recurring likelihood is low. The mesh is safe and generally well accepted by the body's natural tissues (Perez et al., 2005; Terzi, 2006). Polypropylene grafts are commonly used in hernia repair and abdominal wall reconstruction because of decreasing hernia's recurrence and simple application. Mesh repair of abdominal wall hernias has become an integral part of general surgery, currently representing the standard procedure for all kinds of abdominal wall hernia especially in recurrent and incisional hernia repair (Agalar et al., 2006; Edmiston et al., 2006; Merritt et al., 1999). Although rarely seen, infection after a mesh hernia repair may be a challenging problem (Parviz and Amid, 2005; Robinson et al., 2005; Wantz, 1996; Lichtenstein et al., 1990). Whereas data concerning the efficacy of systemic antibiotic prophylaxis remains controversial, the application of local antibiotics has been described as a safe and effective way for the prevention of possible infection. As more than one million mesh implants are used per year globally, improvement of the materials to minimize infection would be beneficial (Gilbert and Felton, 1993; Kilic et al., 2007).

The aim of the present study was to evaluate the antibacterial effect of triclosan coated polypropylene grafts against simultaneously inoculated *S. aureus* (inoculum size is 1.1×10^9 colony-forming units (CFU)/mL) graft infection model. For this coating purpose, triclosan loaded chitosan gels were designed to facilitate the coating procedure with a bioadhesive gel. It was aimed at the same time to provide a favorable release profile for the active drug triclosan when incorporated into a gel system which slows down the drug release process and to provide an efficient therapy for hernia repair.

2. Materials and methods

2.1. Materials

Triclosan was supplied from Veser Kimyevi Maddeler, Turkey and is depicted in Fig. 1. Chitosans (low, medium and high molecular weight) were supplied from Fluka, Buchs, Switzerland. Glacial acetic acid (99–100%) was obtained from Merck, Darmstadt, Germany. All other reagents were of analytical grade and used without further purification.

2.2. Methods

2.2.1. Preparation of chitosan gels

Three different chitosan gel formulations were prepared as follows; 1% (w/v) chitosan (low, medium or high molecular weight) were dissolved in 1% (v/v) acetic acid solution until a clear gel was obtained after overnight stirring. Then, 1% (w/v) triclosan was dispersed in this gel (Hernandez-Richter et al., 2000). Polypropylene grafts were cut into 2 cm × 2 cm squares, incubated in gels for 24 h at room temperature and dried in a vacuum oven.

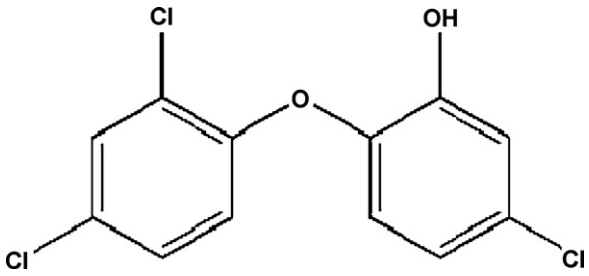

Fig. 1. Schematic representation of triclosan.

2.2.2. In vitro drug release

Release profiles of triclosan from grafts were determined in 100 mL of isotonic PBS (pH 7.4) containing 1% sodium dodecyl sulfate (SDS) providing sink conditions in a thermostated shaker bath system (Memmert, Schwabach, Germany) at 37 °C. The aqueous solubility of triclosan is only about 10 μg/mL. At predetermined time intervals, samples were withdrawn from the system and replaced with equal volume of fresh release medium maintained at the same temperature. The released amount of triclosan was determined by a spectrophotometric assay at 281 nm using a Shimadzu UV–VIS 160A spectrophotometer ($r^2 = 0.999$).

2.2.3. Preparation of bacteria suspension and contamination of grafts

S. aureus ATCC 29213 were plated onto the 5% sheep blood agar and incubated 24 h at 37 °C. After incubation, the colonies were removed to brain–heart infusion broth 3.0 McFarland (1.1×10^9 CFU/mL). Polypropylene grafts were contaminated with 100 μL bacteria suspensions using micropipette before the surgery.

2.2.4. Determination of Minimum Inhibitory Concentration (MIC)

Broth microdilution method was used for the determination of MIC values. The widely used procedure was recommended by the National Committee for Clinical Laboratories (NCCLS). All studies were completed using Mueller Hinton Broth. Minimum inhibitory concentration of microorganisms was evaluated against *S. aureus* ATCC29213, inhibiting the growth of this bacterium concentrations of 2×10^7 CFU/mL, with triclosan concentrations up to 10^{-15} of the initial concentrations (10 mg/mL) (Andrews, 2001; Hernandez-Richter et al., 2000). For this reason, triclosan stock solution was prepared (10 mg/mL). Serial dilutions were made until 10^{-15} with Mueller Hinton broth. Every diluent was put into tubes (100 μL) and added 900 μL *S. aureus* ATCC29213 until 2×10^7 CFU/mL. Every tube was incubated 24 h at 37 °C.

2.2.5. In vivo studies

In the light of in vitro release data, optimum formulation selected for in vivo studies was high molecular weight chitosan gel containing triclosan. This type of chitosan was selected because it was capable of releasing higher amounts of triclosan over the 7-day release period. Thirty-two Wistar albino male rats were divided into four groups (Table 1). Groups were blank grafts (group 1), blank grafts with antibiotic prophylaxis (group 2), grafts coated with only chitosan (group 3), grafts coated with triclosan loaded chitosan gel (group 4). In vivo research has been granted permission by Hacettepe University Ethics Committee (2007/40-2).

All rats were anaesthetized with i.m. xylasine (10 mg/kg) and ketamine (90 mg/kg). Sterility measures were strictly undertaken.

Table 1
In vivo study groups.

Groups	Drugs	i.p. antibiotic	Rat (n)
Group 1	Blank graft	No	8
Group 2	Blank graft	Teicoplanin	8
Group 3	Chitosan	No	8
Group 4	Chitosan + triclosan	No	8

The skin was cleaned with povidone and allowed to dry for 2 min. The inguinal region was exposed and a transverse 1 cm skin incision was made. Grafts were inserted under the external oblique fascia and fixed to the muscle with two separate 4.0 polypropylene stitches. Peritoneum was not opened in any of the animals. The skin incision was then closed by 4.0 polypropylene-interrupted sutures. Incisions were observed to detect macroscopic findings of infection, such as seroma formation, wound dehiscence, hyperaemia and purulent drainage (Gilbert and Felton, 1993; Kilic et al., 2007; Agalar et al., 2006).

On the eighth day, the animals were sacrificed and the grafts were removed. The grafts were put into 2 mL brain–heart infusion broth and been crushed with tissue crushing. The suspensions were diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} of the initial concentrations. The isolated suspensions (100 μL) were cultured on 5% sheep blood agar plates. All plates were incubated 24 h at 37 °C. Then colony-forming units were counted overnight.

2.2.6. Surface morphology of grafts

A scanning electron microscope (Jeol-SEM ASID-10, Device in 80 KV, Japan) was used to evaluate surface characteristics of grafts. Grafts were mounted on the metal stubs with conductive silver paint and then mounted with a 150 Å thick layer of gold in a Bio-Rad apparatus.

3. Results and discussion

In this study chitosan gel delivery system for the antibacterial drug, triclosan was designed and developed. In this context, three different chitosan gel formulations with low, medium or high molecular weight chitosan have been designed and evaluated for in vitro and in vivo properties. For this purpose, in vitro drug release from gels, contamination of grafts with bacteria suspension, determination of minimum inhibitory concentration, in vivo studies and surface morphology of the grafts were evaluated. In literature, triclosan was not applied to polypropylene grafts previously in hernia repair and this was the first experimental study with triclosan coated polypropylene grafts. In the present study, the aim was to reduce or prevent the bacterial adherence to the grafts with the antibacterial effect of triclosan.

Chitosan is a prospective cationic polysaccharide which has shown number of functions in many fields, including biomedicinal, pharmaceutical, preservative, microbial and other properties. Polymeric materials like peptides, polysaccharides and other natural products have recently attracted attention as biodegradable drug carriers. They can optimize clinical drug application, minimize the undesirable drug properties and improve drug efficiency. They are used for the slow release of effective components as depot forms, to improve membrane permeability, solubility and site-specific targeting (Alves and Mano, 2008). Chitosan microspheres and gels seem highly suitable for use in implantable drug delivery. These might be the explanations of preparing chitosan gels for this study.

In vitro release experiments were realized on all gel formulations. Fig. 2 represents the in vitro release profiles of triclosan loaded gel formulations prepared with chitosan (low, medium and high molecular weight). The release was determined to be 4, 5 and 8 μg/mL for medium, low and high molecular weight chitosans, respectively. Most of the drug was released within 24 h. Complete release of triclosan was realized within a period of up to more than 7 days for all formulations. More concentrated gels were reported to dissolve at a slower rate than less concentrated ones because of the decreased water diffusion coefficient for the rate of water diffusing into the gel (Boulmedarat et al., 2003). Release from the gel is, on the other hand, a competitive process controlled by diffusion of the drug upon dilution and competitive displacement of the drug by components in the dissolution media. These two factors might have contributed to the release of triclosan from the gel (Bilensoy et al., 2007). Subsequently, chitosan gel was used as a limiting factor for the release of triclosan from the grafts to the surrounding bioenvironment. Up to this consideration it is clear that, this bioenvironment would have a significantly smaller volume, the release could be expected to be slower in vivo. Following the microbiological studies, MIC value was found 1×10^{-12} mg/mL for triclosan, an important indication of MIC values being at adequate concentrations throughout the release. As the released drug quantities for all formulations were above this value throughout the release studies, it could be concluded that the required MIC value for the inhibition of microorganisms at this concentration is available and provided for in vitro conditions and in vivo evaluation seems to confirm this situation (Table 2). Triclosan quantities were 4, 5 and 8 μg/mL for medium, low and high molecular weight chitosans, respectively. This MIC value is also in accordance with the value reported previously by Hernandez-Richter et al. (2000).

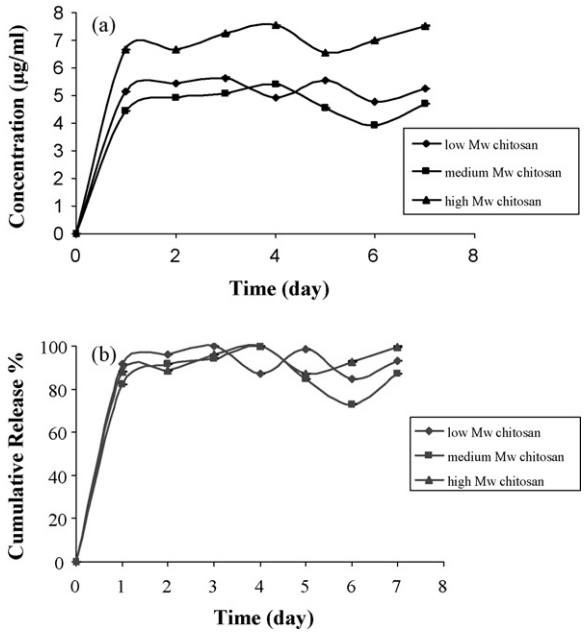

Fig. 2. In vitro release profile of triclosan from chitosan gel coated grafts ($n = 3 \pm$ SD) ((a) triclosan concentration μg/mL, (b) cumulative triclosan release %).

Table 2
Colony counting data of grafts that were simultaneously inoculated (CFU mean values).

Group 1	Group 2	Group 3	Group 4
1000	20	200	5
1500	20	250	0
1500	200	100	6
2000	80	150	0
3000	120	60	10
2800	50	500	2
3000	100	400	0
4000	200	350	3

In literature numerous models have focused on to study for simulations of various clinical situations. These models differ from each other mainly in selecting the responsible organism, foreign material, timing of the infection with respect to the implantation of the material and site of implantation. These differences in study designs cause some problems in applications of these models to clinical situations. In our study, we used polypropylene graft incubation model to study foreign body infection. This method is a simple and reproducible experimental model. In this model we achieved 100% infection rate in untreated rats. Any death, septicaemia and remote organ infection were shown with these rats. In this study, *S. aureus* were selected for the graft infection agents. *S. aureus* are reported to be the major pathogens associated with biomaterial-induced infections. In literature, the advantages of a local antibiotic prophylaxis in hernia repair has been reported extensively (Taylor et al., 1997; Lazorthes et al., 1992; Musella et al., 2001; Zimmerli et al., 1984) and stated that the routine use of mesh materials seems to have the similar advantages as antibiotic loaded implants. In this study, simultaneous bacteria inoculation was used to model mesh infection before the surgery to provide the standard volume of bacteria suspensions.

No death occurred in any of the groups during in vivo study. Macroscopic findings of infection were detected in all rats in groups 1 and 3 at the end of the study period. However, graft infection was detected in five (62%) of the rats in the group 2. Wounds were completely separated in rats of these three groups. It was observed that there was not any abscess in group 4. For group 1, wound collection and purulent incisions, for groups 2 and 3 wound collection and necrosis were observed. There were not any wound collection, purulent incisions and necrosis detected in group 4. Macroscopic findings of groups are presented in Fig. 3.

The bacterial growth was not detected on group 4 grafts, whereas the highest rate of growth was found for group 1. The amount of bacteria detected in groups 2 and 3 were also significantly less than group 1. The mean CFU values of groups were 2400, 90, 225, 2.5 for groups 1, 2, 3 and 4, respectively. Results were depicted in Table 2. All in vivo study groups were comparatively evaluated in terms of bacterial adherence of grafts. Triclosan

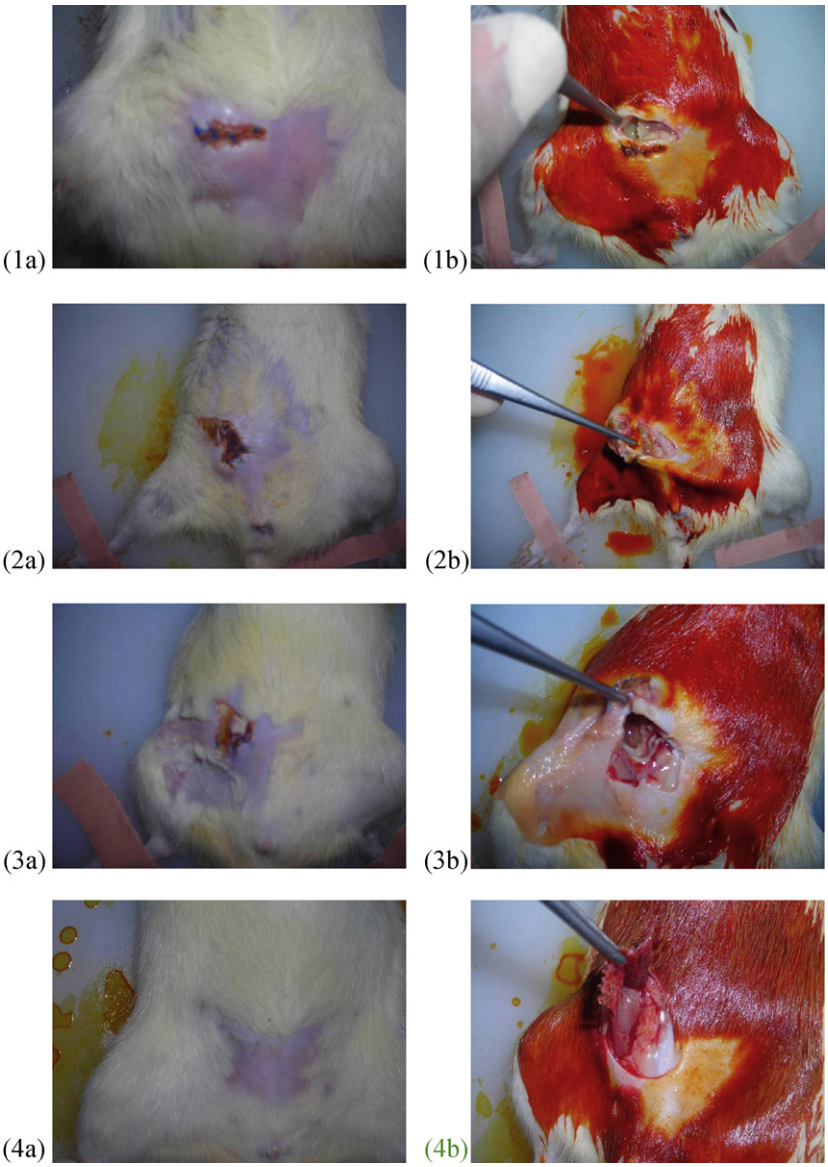

Fig. 3. Macroscopic findings of groups [(1a) wound collection and purulent incisions (group 1—blank graft/no antibiotic); (1b) abscess, 8 days later (group 1—blank graft/no antibiotic); (2a) wound collection, necrosis and purulent incisions (group 2—blank graft/i.p. teicoplanin); (2b) abscess, 8 days later (group 2—blank graft/i.p. teicoplanin); (3a) wound collection and necrosis (group 3—chitosan/no antibiotic); (3b) abscess, 8 days later (group 3—chitosan/no antibiotic); (4a) no wound collection, necrosis and purulent incisions (group 4—chitosan:triclosan/no antibiotic), (4b) no abscess, 8 days later (group 4—chitosan:triclosan/no antibiotic)].

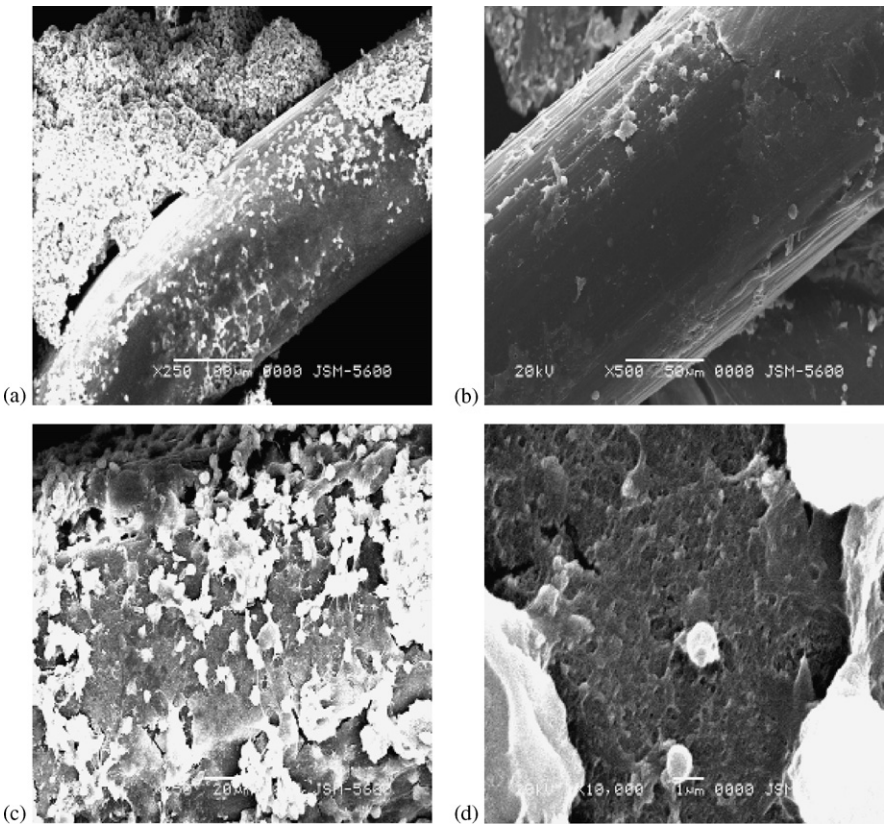

Fig. 4. SEM photographs of the grafts ((a) group 1—blank graft/no antibiotic; (b) group 2—blank graft/i.p. teicoplanin; (c) group 3—chitosan/no antibiotic; (d) group 4—chitosan:triclosan/no antibiotic).

loaded chitosan gels (group 4) were reduced the bacterial adherence 960 times less than the blank grafts (group 1). Also, triclosan loaded chitosan gels (group 4) reduced the bacterial adherence 36 times less than when compared to the prophylaxis group (group 2) and 90 times less than the blank chitosan gel group (group 3). These results were confirmed with SEM photographs of the grafts. In vitro drug release studies show us that drug quantity in the media was over the MIC value and it was adequate to prevent the bacterial infection.

Imaging of the grafts by SEM was expected to provide information on morphology of the grafts. SEM images of the grafts are presented in Fig. 4. Examination of SEM photographs of the grafts revealed that group 4 significantly reduced the bacterial adherence when compared with groups 1, 2 and 3 which was the aim of this study.

4. Conclusion

The findings of the present study indicate that the polypropylene graft incubation model is a simple and reproducible experimental model in hernia repair. The fact that 100% infection rate was achieved in untreated rats is an indication for this. This study revealed that triclosan–chitosan coated grafts were effective against the reduction of bacterial adherence to polypropylene grafts and further graft infection. It could be concluded from our experimental studies that, for treatment of hernia repair, triclosan coated grafts were effective enough and additional prophylactic antibiotics is not required. Chitosan gel was found to be a favorable agent for the coating of polypropylene grafts with an antibacterial agent in terms of its physicochemical and biological attributes.

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