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MECHANISMS INVOLVED IN THE EFFECT OF CHITOSAN IN ENHANCING HEALING

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MECHANISMS INVOLVED IN THE EFFECT OF CHITOSAN IN ENHANCIING HEALING

By

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ABSTRACT

MECHANISMS INVOLVED IN THE EFFECT OF CHITOSAN IN ENHANCIING HEALING

By

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Chitosan is a natural biopolymer that consists of a poly β (1-4) glucosamine. It has been described as being a wound healing accelerator. In this study, two approaches were taken to investigate the biological basis of wound healing enhancement by chitosan. Firstly, the antibacterial effect of chitosan was tested in-vitro, focusing on two bacteria strains, *E.coli* and *S.aureus* using the microbroth dilution assay and the gel diffusion test. The microbroth assay showed that chitosan and the acetic acid solvent had the same minimum inhibitory concentration (MIC) ranges. Likewise, no zone of growth inhibition was detected by gel diffusion test. In the second experimental approach, the cellular response to the intra-peritoneal implantation of chitosan pads in August rats was defined over time and compared to Gelfoam®. In-vivo, as early as 4 hours post-implantation, the cellular response induced by chitosan was significantly more than that to the Gellfoam®. However, the reaction declined with time with chitosan, but continued to increase to the Gellfoam ® pads. After two weeks, there was minimum collagen deposition, i.e. minimal scarring, associated with chitosan pads compared to that of the Gelfoam pads. The cellular response to chitosan was seen to include cells of the immune system. These results support the concept that chitosan enhances wound healing.

To my Mother, Aziza
&
To my brothers and sisters

Without their love and sustained support,
This could not be achieved

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TABLE OF CONTENTS

LIST OF TABLES

LISTS OF FIGURES

"Images in this thesis/dissertation are presented in color"

CHAPTER 1.

LITERATURE REVIEW AND OBJECTIVES

LITERATURE REVIEW

Chitin was first described by the French scientist, Braconnot (1811), when he isolated it from mushrooms using diluted alkali; he called it "fungine". Later, Odier (1823) isolated the same substance from insects and called it chitin, using the Greek word for "tunic envelope". Rouget (1859) isolated chitosan by boiling chitin in a very concentrated solution of potassium hydroxide, the product being soluble in organic acids (Muzzarelli 1977). The natural deacetylated form of chitosan was identified in 1954 from the cell walls of the fungus Phycomyces blackesleeanus (Kreger 1954). It was also found in the walls of Zygomycete soil fungi and in green algae such as Chlorella ellipsoidea (Mihara, 1961).

Chitin:

Chitin is widely abundant in nature and is second only to cellulose in availability. It is mainly found as a component of the exoskeletons of crustaceans and insects, as well as in the cell walls of some bacteria, fungi, algae, hydrozoa, annelida, molluscans and nematode worms (Muzzarelli 1977). The proportion of chitin present is different for the various species (Table 1). The traditional and commercial sources of chitin are the shells of crab, shrimp and krill, all of which are wastes from marine food processing. The worldwide annual production of crustacean shells has estimated to be 1.2×10^6 tons and the production of chitin and protein from this waste can be considered as a major additional source of commercial income (Knorr 1991).

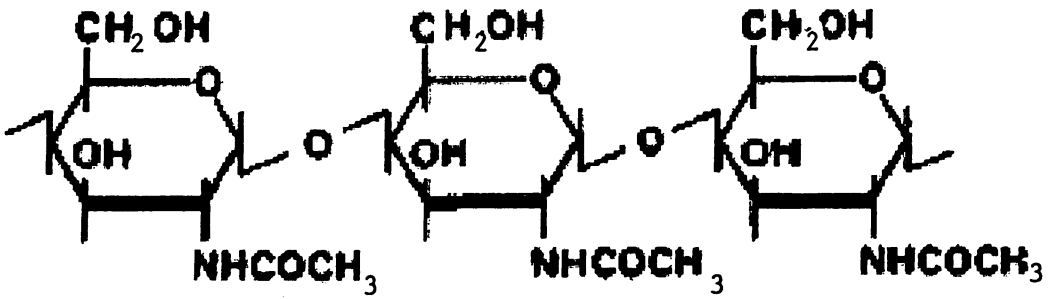
Table 1. Chitin content and types from different sources.

(Modified from Rhazi et al. 2000 and Rudrapatnam et al. 2003)

Chitin source	Chitin content (%)	Chitin source	Chitin content (%)
Crustacea:		**Insects:**	
Marbled crab	10	Blatella (cockroach)	18.4
Red crab	10	Colcoptera (beetle)	27-35
Spider crab	16	Bombyx (silk worm)	44.2
Cancer crab	72.1	Calleria (wax worm)	33.7
Carcinus crab	64.2	**Molluscan organs**:	
Lobster	17	Clam shell	6.1
Locust lobster	25	Oyster shell	3.6
Spiny lobster	32	Sguid, skeletal pen	41
Homarus lobster	60-70	Krill	40
Crayfish	36	**Fungi:**	
Shrimp	22	Aspergillus niger	42
Squilla	24	Pencillium notatum	18.5
Cephalopda:		Sacharomyces cereviseae	2.9
Cuttlefish	20	Mucor rouxii	44.5
Squid	40	Mushroom	19

Chitin is a white nitrogenous linear polysaccharide polymer, consists of poly ß- (1-4) linked N-acetyl-D-glucosamine with a molecular weight reaching millions of Daltons (Figure 1). It is tough, relatively inert, and insoluble in water and in organic acids. However, most of the different forms of chitin are soluble in various concentrations of chloro-alcohols conjugated with mineral acids or organic acid solutions (Austin 1975).

Figure 1.The structure of chitin (after Mochizuki et al.1989)



Chitin is present in three different crystallographic forms,α , β, and γ, which differ in the arrangement of the polysaccharide molecular chains within the crystal cells (Rudall, 1963). The chains of α-chitin are arranged in anti-parallel manner with strong intermolecular hydrogen bonding (Minke et al. 1978). The β-chitin is characterized by presence of parallel chains with relatively weak intermolecular forces (Gardner and Blackwell 1975). The γ - chitin has one chain directed down after every two chains directed up. The most abundant and stable form is the α – chitin (Minke et al. 1978) which is commonly found in crab and shrimp shells. Most of crustaceans have α– chitin while cephalopods contain β-chitin form (Rhazi et al. 2000).

Currently, the chemical method used for commercial production of chitin is a thermo-chemical process based on demineralization and de-proteination of shellfish waste (Roberts 1997). Due to various drawbacks of the chemical method, an alternative biological method has recently been utilized by some

processors, and is found to be efficient and readily applicable (Jung et al. 2006). This is based on the fermentation of shell wastes with proteolytic microorganisms e.g. Lactobacillus paracasei subsp, tolerans KCTC-3074, and Serratia marcescens FS-3.

Chitosan:

Chitosan is semi crystalline poly ß - (1-4) – glucosamine with molecular weight varies from about 10,000 to 2 million Daltons (Figure 2). It is produced from chitin by deacetylation (Figure 3). Chitosan preparations can vary considerably in the degree of acetylation, solubility and the molecular weight.

Figure 2. The structure of Chitosan (after Mochizuki et al. 1989)

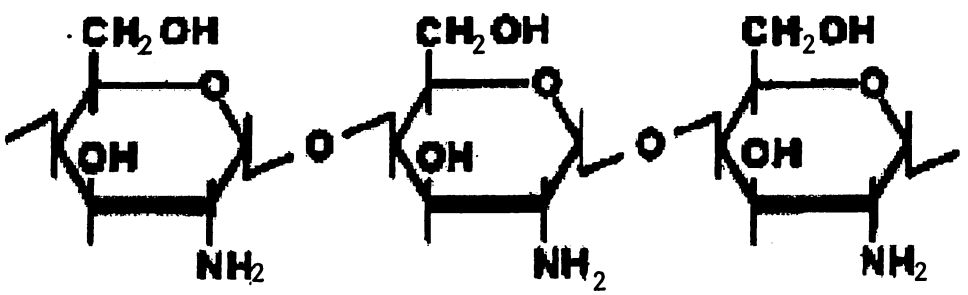
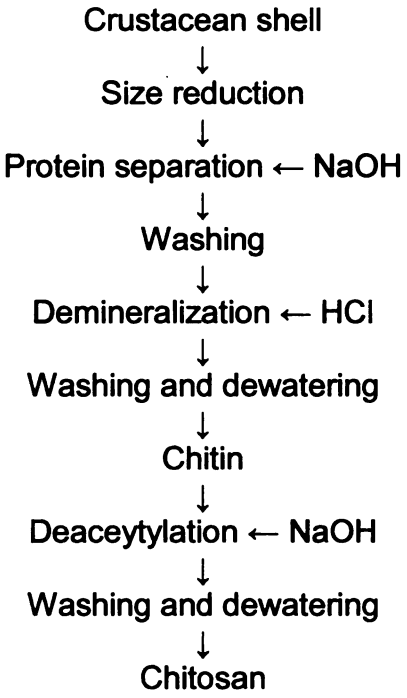


Figure 3. Production of chitin and chitosan (after Zikakis 1984)



Deacetylation of chitin to produce chitosan is performed either by using strong alkai (Roberts 1997) or chitin deacetylase enzyme, e.g. fungal chitin deacetylases (Tsigos et al. 2000). The chemical method has disadvantages of both consuming considerable amount of energy and using a large amount of concentrated alkali that can lead to environmental pollution and production of impure heterogeneous compounds. On the other hand, the deacetylase method results in the production of novel, chemically and physically, well-defined chitosan oligomers and polymers which attain the prerequisite quality required for this compound's many medical uses.

Chitosan and chitin can be fabricated in many forms such as powder, gels, flakes, membranes and sponges. A major physical difference between chitin and chitosan lies in their solublization, which is controlled by the degree of acetylation (DA). The DA is defined as the molar fraction of N-acetyl-glucosamine residues with respect to total units. In general, when the DA is <60% the polymer is termed chitosan and when it is >60% the polymer is called chitin (Sorlier et al. 2003).

Chitosan is highly basic polysaccharide that is insoluble in neutral and alkaline pH, but forms soluble salts with inorganic and organic acids such as glutamic, hydrochloric, lactic and acetic acids. Different degrees of deacetylation or depolymerization produce different physiochemical properties, and by changing the degree of deacetylation the solubility of chitosan is also modified. Another characteristic of chitosan is its high viscosity in an acidic environment. The level of viscosity of chitosan is influenced by the molecular weight, concentration, degree of deacetylation, ionic strength, pH and the temperature (Wang and Xu 1994) (Table 2). Moreover, both chitin and chitosan have high nitrogen content (6.89%), which allows them to act as chelating agents (Rabea et al. 2003).

Table 2. Chemical characteristics of chitosan from different sources.

(Modified from Rhazi et al. 2000)

Chitin source	Yield of chitosan from chitin wt (%)	Intrinsic viscosity (ml g^{-1})	Average molecular weights M_v (g mol^{-1})	Degree of Acetylation (%) (NMR)
Barnacle	78	357	66 000	13
Marbled crab	65	313	55 000	4
Red crab	76	315	56 000	9
Spider crab	75	266	45 000	3
Lobster	77	384	72 000	10
Locust lobster	77	485	98 000	16
Spiny lobster	78	422	82 000	12
Crayfish	79	426	83 000	8
Shrimp	78	582	125 000	10
Squilla	67	429	84 000	4
Cuttlefish	64	74	8300	3
Squid	70	128	17 000	0.5

Uses of chitosan:

By changing the degree of acetylation of chitin and the level of viscosity, a wide range of chitosan forms can be produced. These different forms display diverse potential uses in the industrial and medical fields. Commercially, due to its cationic nature, chitosan is used to purify water from heavy metals and pesticides (Onsoyen and Skaugrud 1990). In agriculture it has been used to coat seeds of the plants as well as directly as a fertilizer that results in an increase of crop yields (Hadwiger et al. 1984). Also, it used as an animal feed additive due to its high dietary fiber content and its hypocholesterolemic effect (Rudrapantam et al. 2003). In addition, due to its high viscosity, film-forming and the moisture

retention properties, chitosan has been used in cosmetic industry products such as hair shampoo and conditioner (Chen & Heh 2000).

Medical applications:

Chitosan is a physically and biologically functional compound characterized by lack of toxicity and allergenicity. In addition, it is biocompatible and biodegradable and can be modified chemically and enzymatically. All these features provide a stimulus for investigators to devise methods of utilizing chitosan for various medical and pharmaceutical applications. For example, it is used as a wound-healing agent, an antimicrobial, a haemostatic agent, and as a medium for drug and vaccine delivery. (Loke et al. 2000, Mackenzie et al 2006, Ueno et 2001, Illum et al 2000).

1. Antimicrobial activity:

Most of the evidence of the antimicrobial activity of chitosan is reported with food and water borne microorganisms and is associated with the use of chitosan as plant protectants and food preservatives (Taha et al. 2002). The antibacterial effect was found against several strains of bacteria, including *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus*, and *Salmonella typhimurium* (Sudarshan et al 1992, Rabea et al. 2003) Generally, chitosan is thought to show a more powerful bactericidal effect against gram-positive bacteria than gram-negative bacteria (Senel & McClure 2004). Chitosan also has antifungal effect,

and is found to be effective against *Candida albicans* and *F. oxysporium* (Tsai 2002).

Chitosan is also used as a wound dressing and has shown to control the bacterial multiplication and invasion in wound injuries (Loke et al. 2000, Mi et al. 2001). Loke et al. (2000) formulated a chitosan dressing consisting of two layers; chitosan hydrogel in the upper layer and an anti-microbial impregnated biomaterial in the lower layer. An inhibition zone around discs of chitosan acetate incorporated with chlorehexidine gluconate was observed and the antimicrobial capability of the dressing was found to be more effective against gram-positive bacteria than the gram- negative ones. Similarly, Mi et al. (2001), carried out an in vivo study using chitosan incorporated with silver sulfadiazine in rats injected with *Pseudomonas aeruginosa* and *Staphlycoccus aureus* strains. A significant decrease in the number of colony-forming units was observed in the tissues surrounding the infected wound. Thus chitosan membranes have the capability of preventing the penetration of the bacteria into wounds and acts as a prophylaxis against wound sepsis (Mi et al. 2001).

2. Wound healing:

The use of chitosan and chitin in wound healing goes back to ancient times. Koreans used the pen of the octopus as a source of chitin for the treatment of abrasions, and Mexicans used mushroom to accelerate the healing of wounds (Allan et al.1984). In current times, the use of chitin as a wound-healing accelerator begins with the studies of Prudden et al. (1957, 1970) who

noticed that shark cartilage promote wound healing and stated that the N-acetyl glucosamine units (GlcNac) play the major role in healing.

Chitosan has many physiochemical properties that make it a good candidate for wound healing. When chitin and chitosan incorporated with other materials, they can form tough, water absorbent biocompatible and interactive films that are capable of absorbing the exudates generated from wounds (Seo et al. 2000). Also, they are characterized by high water vapor transmission, which prevents the accumulation of fluids in wounds. When chitosan membranes are applied to human skin, they adhere uniformly to the wound surface, and prevent formation of air pockets and the accumulation of exudate fluid (Azad et al. 2003). Chitosan has high oxygen permeability ($7x10^{-11} cm^2/s$), which reduces oxygen deprivation of the tissues, and it is slowly degraded by lysozymes that are transported to the wound area by various inflammatory cells (Allan, et al 1984).

Chitosan induces an inflammatory response and enhance leukocyte migration and accumulation of neutrophils and lymphocytes (Hidaka et al 1999). It also stimulates the production of interlukin 8 (IL-8) which chemoattracts and recruits neutrophils to wounds (Mori et al. 1997). Chitosan scaffold implanted intra-peritoneally and subcutaneously in mice did not induce any pathological inflammatory tissue response to chitosan such as erythema and edema, but there was a chemotactic effect on neutrophils (VandeVord et al. 2001). Also, Chellat et al. (2000) demonstrated that after the implantation no signs of cell damage such as swollen mitochondria and rough endoplasmic reticulum , are seen.

Additionally, chitosan stimulates macrophages to produce more essential cytokines which may speed up the wound healing (Mori et al. 1997, Nishimura et al.1986). Ueno et al (1999) in their experiments with beagle dogs, found infiltration of PMN and migration of macrophages and giant cells at an early stage, as well as an increase in the phagocytic activity and the oxidative burst of macrophages and PMN. The activation of peritoneal macrophages by chitosan was indicated by the presence of activation markers including MHC I and MHC II, Fc and mannose receptors.

The second phase of natural wound healing is characterized by the presence of granulation tissue followed by the proliferation of fibroblasts and production of ground substances and extracellular matrix (ECM) filling of the dead spaces. Chitosan accelerates the reformation of connective tissues and angiogenesis of the healing wound (Muzzarelli et al. 1988). Open wounds heal by the initiation of an acute inflammatory phase and cellular response, which then leads to granulation of tissue formation, re-epithelization and wound contraction (Adam & Clark 1999). Both chitin and chitosan enhance the proliferation of fibroblasts and keratinocytes (Howeling et al. 2001). Likewise, Azad et al. (2003) observed abundant keratinocytes in the areas dressed with chitosan membrane with a resultant faster re-epithelization of wounds. Histologically, most of the tissue found after 7 days of implantation of chitin and chitosan is thought to be granulation tissue (Kojima et al. 2004).

Synthesis of collagen, and the formation and remodeling of the extracellular matrix, characterize the third phase of wound healing. Chitosan

promotes the tensile strength of tissue by speeding up the formation of fibroblasts and the synthesis of collagen with more thickly and consolidated collagen fibers (Azad et al. 2003). Lysozymes slowly hydrolyze chitosan membranes and produce chito-oligmers that stimulate deposition, and the assembly of collagen fibrils in the extracellular matrix components (Muzzarelli et al. 1999). An increase in collagen synthesis associated with chitin and chitosan application has been measured using prolyl-hydroxylase enzyme (PHL) activity (Kojima et al. 2004).

3- Anticoagulant properties:

Both chitin and chitosan show a haemostatic activity when applied in bleeding wounds and their muco-adhesive nature make them ideal haemostatic agents (Evans et al. 1962). The contact of GlcNAc derivatives with platelets leads to their activation, aggregation and a change of morphology that suggested the irreversible activation of platelets (Thatte et al, 2004). Chou et al. (2003) demonstrated that the chitosan enhancement of platelet adhesion and aggregation varies with both time and dose. This aggregation is accompanied by an increase of calcium level and expression of glycoprotein IIIb/IIIa complexes on the platelet membranes and leads to interaction of platelets with damaged tissues and the promotion of wound healing.

A freeze-dried chitosan-based hemostatic dressing can control severe parenchymal and large venous hemorrhage in a swine model with liver injury (Pusateri et al. 2003). Recently, chitosan-based haemostatic dressings were

used to reduce hemorrhage in military forces in Iraq and Afghanistan (Wedmore et al. 2006). The dressings were applied to wounds in extremities, chest and groin. In 97% of the cases the bleeding was stopped or greatly improved. Various hypothesis were put forward to explain why chitosan enhances clotting, Chou et al. (2003) suggested that chitosan dressing enhances platelet function, while Klokkevold et al. (1991) suggested the incorporation of red blood cells into the clot was the key. However, Wedmore et al. (2006) reported that the main factor by which chitosan enhances clotting, is its muco-adhesive property.

4- Delivery of drugs and vaccines:

Chitosan microparticles have great potential in mucosal vaccine delivery systems and potentially have adjuvant activities. Suspensions of microparticles have shown an immuno-stimulatory activity in macrophage proliferation, in tumor growth suppression, in cytokine inducement and antibody production. They have the ability to deliver drugs across the mucosal lining, and with their cationic muco-adhesive property, they interacts with negatively charged cell surfaces and cause redistribution of protein zones enhancing the permeability of epithelia to the uptake of peptides and proteins, and thus increasing the contact of such antigens with the immune system (Illum et al. 2000; Hwang et al. 2000).

Chitosan has been used as adjuvant to enhance the immune response elicited to a number of human and veterinary vaccine antigens. Chitosan stimulates non-specific resistance against Sendi virus, and *E. coli* when given prior to challenge infection of mice. The intranasal and intravenous

administration of chitosan as an adjuvant was seen to be more effective than the subcutaneous or intra-peritoneal administration. It appears to act early on the non-specific elements of the immune response such as the accumulation and activation of macrophages, the activation of natural killer cells and the induction of IFN- γ (Iida et al. 1987). The 70% deacetylated chitin stimulates the production of monokines such as CSF, IL-1 and TNF-α, and this adjuvant activity may account for the induction of delayed hypersensitivity, circulating antibody and cytotoxic T-lymphocytes (Nishimura et al. 1986).

In a study carried out on BALB/c mice injected with recombinant protein beta-human chorionic gonadotropin (rβhCG) combined with two different formulations of chitosan, more than a 100- fold increase in antibodies titers was achieved; the chitosan also primed the animals for a specific DTH response (Seferian & Martinez 2001). Strong augmentation of both systemic and local immune responses, with significant enhancement of IgG production, was also seen when mice were vaccinated orally and nasally with Diptheria toxoid (DT) incorporated with chitosan (Inez et al. 2003). Chitosan fed to rats elicits the release of IL-10 and the expression of IL-4 and TGF-β mRNA in mucosal tissues and Peyer's patches, as well as with the activation of $CD3^+$ T cells in the spleen (Porporatto et al. 2005).

Immune response:

Chitin with its rigid crystalline structure has the potential to stimulate the immune system when they carry certain chemical groups. For example, the

residual amino group, as well as the imino derivatives of chitosan, may stimulate the immune system despite the low pK of the amino group of chitosan, which ranges between 6.3-6.4 (Tokura et al.1999). Normally the implantation of the polymer leads to inflammation; however a moderate inflammation may be useful. During the inflammatory reaction the production of specific enzymes and NO by macrophage and other cells augment polymer degradation and allow normal growth of the tissues (Peluso et al. 1994).

Various research groups have reported properties of chitin and chitosan to stimulate immune responses. The degree of deacetylation affects the stimulatory activity of chitin and chitosan (Hidaka et al.1999) . Nishimura et al. (1984) showed that mice peritoneal macrophages were activated when chitin of different degrees of deacetylation and substituting groups were injected intra-peritoneally. Macrophage activation indicators such as the percentage of lysis, stasis and the release of H_2O_2 were found to be highest with 70 % DDA chitin. The activation induced by carbomethyl-chitin is same as the 70% DDA chitin, whilst phosphorlyated and sulphonated-chitin did not activate macrophages, and this suggested that the free amino group likely plays the major role in activating the peritoneal macrophages.

The activation of macrophages is important in host immune responses, but their uncontrolled activation may lead to septic shock and death (Car et al. 1994). To control the unlimited inflammation and activation of macrophages, chitosan induces Fas-mediated apoptosis of the macrophages of BALB/c mice

through the mannose receptor, which may be a mechanism by which it enhances wound healing (Mori et al. 2005).

Chitosan also has an effect on the synthesis of nitric oxide (NO). When macrophages stimulated with combination of chitosan and IFN-γ, their synthesis of NO, cytotoxicity to tumor cells and their TNF-α secretion are increased (Seo et al. 2000). Macrophages cultured in medium containing chitosan produce an increase amount of nitrate (Pelsuo et al. 1994). The production of NO is mainly attributed to the N-acetylglucosamine (NAGA) unit rather than the glucosamine residues.

Chitosan can enhance the production of the main components of adaptive immunity, as well as cell-mediated and antibody-mediated responses. It induces production of circulating antibodies for the bacterial α-amylase and dinitrophenyl-ovalbumin. 30% DDA chitin and S-chitin were the best among water-soluble derivatives for production of circulating antibodies when used as an adjuvant (Nishimura et al. 1985). Also, chitosan can promote the stimulation of delayed-typed hypersensitivity to (ABA-N-acetyl tyrosine) as measured by skin reaction test; the highest immunogencity was found associated with 70% DAA chitin (Nishimura et al. 1985).

Chitosan affects the production of cytokines. It induces monocytes, through binding to CD14, to produce TNF-α; this effect depends on the molecular weight and the neutral solubility (Otterlei et al. 1994). A high concentration of the poly-ionic chitosan-xanthan (CH-X) complex stimulates macrophages to produce TNF-α and IL-1β that are important mediators of

inflammatory response involved in cell proliferation, differentiation and apoptosis (Chellat et al. 2000).

Degradation of Chitin and chitosan:

One of the main characteristics of chitin and chitosan that favors their use in medical areas is their biodegradability (Tomihata & Ikada 1997). Chitinase is an enzyme, which hydrolyzes chitin to free N-acetylaglucosamine (GlcNAc); it consists of two hydrolases; chitin glycanohydrolase, and chitobiase. These are found in the microorganisms as well as in various plants and animal species (Muzzarelli 1977). Chitinases break down the chitin polymer into intermediate sized chitin oligosaccharides, which then are hydrolyzed to chitobiose with production of small amount of GlcNAc. Finally, chitobiase splits chitobiose into molecules of GlcNAc. Chitosan can be degraded by lysozymes, and related enzymes such as N-acetyl-B-D-glucaminidases, which occur in the human body (Bourbouze et al. 1991). The mannose receptor is the major macrophage receptor for polysaccharides (Marodi et al. 1993). After binding to mannose receptors, chitin and chitosan are internalized by the cell and then degraded by lysozyme to N-acetyl D-glucosamine (Pangburn et al. 1982, Tomihata& Ikada 1997). The rate of chitosan degradation is inversely related to its degree of crystallinity, and the degree of acetylation (Hirano et al. 1989).

Hypothesis and objectives:

The present study addresses two main hypotheses associated with the role and effect of chitosan in wound healing;

Hypothesis 1: Chitosan has antibacterial activity which will enhance wound healing.

Hypothesis 2: Chitosan improves the healing process and reduces the amount of resultant scar tissue.

Two approaches were adopted to test these hypotheses. Firstly, to measure the antibacterial effect of chitosan in vitro against two common bacterial strains associated with wounds, *Escherichia coli* and *Staphylococcus aureus*. The second approach was to define the cellular response to intra-peritoneal implantation of chitosan and compared it with the regularly used material, Gelfoam® (a collagen based material used to prevent intra-peritoneal bleeding and promote healing).

CHAPTER 2.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. Microbiological experiments:

1. 1: Measurement of the antibacterial activity of chitosan by microbroth dilution assay:

Four different compounds were used, MSU 030, MSU 033, (controls), and MSU 034 and MSU 035 (Chitosan) (Table 3) (Vanson Halosource Company). The bactericidal activity of chitosan was examined under varying environmental factors. Factors tested were acid solvent, pH and the mode of sterilization:

Table 3. Characteristics of the microbroth dilution assay samples.

Test sample	Type	DDA	MW (Daltons)	Source
MSU030	2% aqueous gel of hydroxyl propyl methyl cellulose	NA	ND	Crab
MSU 033	Hydroxy propyl methyl cellulose powder	NA	ND	Crab
MSU034	6.6% aqueous solution of ethylene glycol chitosan	<7%	97,000	Crab
MSU035	Ethylene glycol chitosan lyophilized powder	<7%	97,000	Crab

NA: Not applicable , ND: data not available

Acid solvents effect:

To assess the effect of acid solvent, two organic acids were used; acetic acid and formic acid. In the first set of experiments, two concentrations of chitosan were used, 1% and 2%, and they dissolved in either 1% or 2% of acetic acid. In the second set, 2500 ppm chitosan was dissolved in 0.2M formic acid.

pH effect:

The effect of pH was assessed in the experiments where acetic acid was used as the solvent. The experiments were carried out by adjusting the pH to 2.6, 4.2, 5.0, and 6.0. While, in the experiments where formic acid used as solvent, pH 5.0 was used throughout all experiments.

Mode of sterilization effect:

In a set of experiments, chitosan solution was sterilized by autoclaving at 121 ° C for 15 minutes. As a control, a second set of experiments without sterilization was performed.

Preparation of media:

Muellor Hinton II Agar media (MHA): 38/.k grams of the MHA powder (B 11438, Becton Dickinson, MD) was suspended in 1 liter of distilled water, boiled for 1 minute and autoclaved at 121° C for 15 minutes. The mixture was poured into petri dishes to obtain a 3-4 mm deep agar, dried and stored in sealed plastic bags at 4° C.

Muellor Hinton broth (MHB): 22 grams of the MHB powder (211443 Becton Dickinson, MD) were dissolved in 1 liter water heated with frequent agitation and then boiled for 7 min. The melted broth was dispensed into sterile capped bottles, autoclaved for 20 min at 121° C and stored at room temperature.

Growth of bacteria:

A Gram-negative *Escherichia coli* ATCC 25922 strain was grown on MHA media for 20-24 hours at 37° C. The inoculum of the bacteria was prepared by swabbing off the bacteria from the plate and suspending the cells in MHB. The spectrophotometeric absorbance was adjusted to 0.1 O.D. at 560 nm which contains approximately $5x10^8$ CFU/ml. The suspension was further diluted 500 times with MBH before it was used in the assay, at concentration of $1x10^6$ CFU/ml bacteria for use in each plate.

Controls:

Two forms of cellulose were used as controls. In addition, in each assay, two positive controls were used; Kanamycin monosulfate antibiotic (K-4378, Sigma, MO), and a "column" in the plate containing only the bacteria and the broth. Similarly two negative controls were used; 1% acetic acid, and a "column" in the plate containing only the broth.

Preparation of stock antibiotics:

The following equation was applied to determine the weight of Kanamycin antibiotics to be used (NCCLS, 1997)

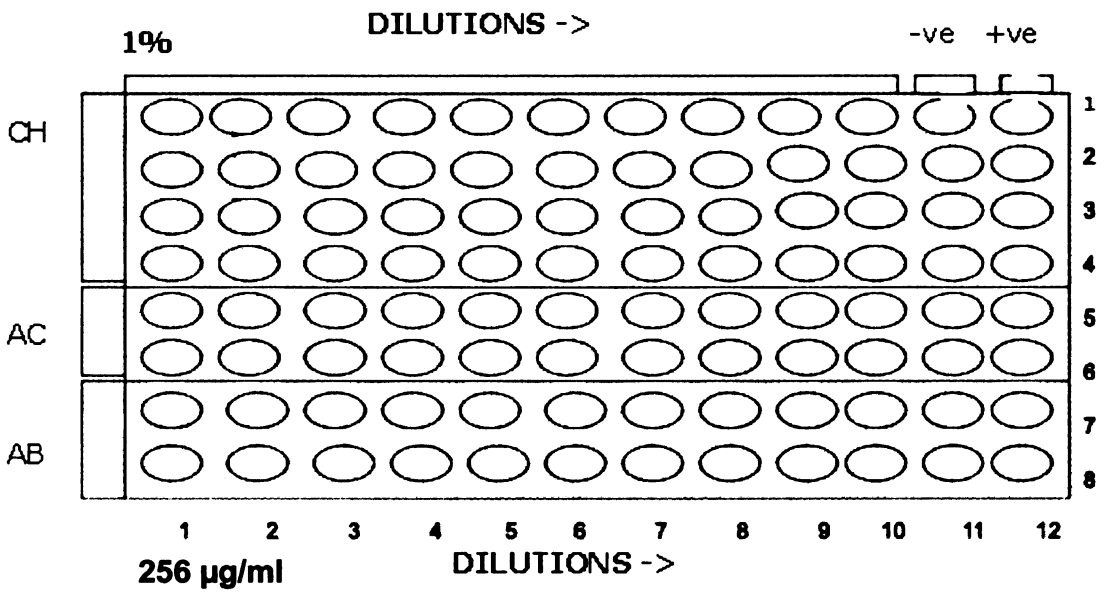
$$\text{Weight (mg)} = \frac{\text{Desired concentration (}\mu\text{g/ml) X volume (ml)}}{\text{Potency of the antibiotic (}\mu\text{g/ml)}}$$

The potency of Kanamycin is 781 µg/ml (Sigma, MO). The powder was dissolved in sterile water, and further diluted to make up the required volume. Stock antibiotics were aliquoted and stored at –80 $^{\circ}$C for no more than 6 months.

Microbroth dilution assay:

96 round-bottom well plates (3799, Corning incorporated Costar, NY) were used to obtain both a qualitative and a quantitative measures of any antibacterial activity of chitosan. Plates were evaluated for growth as indicated by the presence of either turbidity or pellets of the U shaped bottom well after 24 hours. The antibiotics were diluted to twice the required starting concentration with MBH.

Figure 4. Microbroth dilution assay plate design.



Microbroth procedure:

100 µl of 1% chitosan were placed in the first 4 rows of the first column in the plate, followed by 100 µl of 1% acetic acid in rows 5 and 6. In the last two rows, 7 and 8, 100 µl of antibiotics were placed. 50µl of MHB was placed in all of the remaining wells of the plate. Then, 50µl of 1% chitosan were transferred from column 1 to column 2 mixed and so on transferred by serial dilution until reaching column 10 (0.0019% concentration) . Similarly, 50µl of 1% acetic acid and 50µl of antibiotics were transferred by serial dilution until column 10. Finally, 50µl of the bacterial suspension was added to column 12 and then from column 2 till the 10^{th} column. Column 12 is the positive control; it contains the bacterial suspension only. Column 11 is the negative control containing only the broth.

The plate was incubated at 37°C, in 5% CO_2, till growth was seen in column 12, usually after 20-24 hours.

Microbroth analysis:

The lowest concentration of chitosan or the controls where no growth was seen in the well was taken as the minimum inhibitory concentration (MIC). The MIC tested for the antibiotics is within the range of 0.5 μg /ml to 128 μg /m (NCCLS 1997). Images of the plates were taken using a camera with an Alphaimager software program (Version 3.3).

1.2: Agar diffusion assay:

Preparation of chitosan solution:

Amphoteric form of chitosan, chitosan succinate, was dissolved in sterile deionized water, and used at concentrations of 1%, 0.5% and 0.25% in the assay. To desalt the chitosan, the solution was passed through filter paper No.1 and then sterilized by passage through 45 μm filter membrane.

Strains of bacteria:

The gram-negative *Escherichia coli* ATCC 25922 , and the gram-positive *Staphylococcus aureus* ATCC 29213 strains were used in this assay. They were grown on MHA media for 20-24 hours, and then the bacteria suspended in MHB at a concentration of $1x10^8$ CFU/ml throughout the experiments,

Controls:

Kanamycin antibiotic was used as a positive control at a concentration of 30 µg. 1% sodium succinate (S2378, Sigma, and MO) was used as a negative control.

Agar diffusion procedure:

A bacterial suspension at a concentration of $1x10^8$ CFU/m was streaked out on a 100mm x 15mm MHA plate. Susceptibility discs (1599-33 Difco laboratories, Detroit) were used and placed in the plate. The optimal concentration of the chitosan solution needed to saturate the disc was found to be 20 µl. In each assay 20 µl of 1%, 0.5% and 0.25% chitosan were used. In addition, 20 µl of Kanamycin antibiotics and 20 µl of 1% sodium succinate were used as controls in each plate.

Agar diffusion analysis:

The zones of growth inhibition surrounding each disc were measured in each plate after incubation for 18 hours at 37° C. The susceptibility of *E.coli* and S. *aureus* to chitosan was determined to be either resistant (- ve) or sensitive (+ve). Images of the plates were taken using a camera with an Alphaimager software program (Version 3.3)

2. Cellular response to intra-peritoneal implantation of chitosan:

Animals:

The animal models used in this investigation were of August rat inbred strain (Originating at the MRC, London, UK). August rats were used in this study for the following reasons:

1- They are inbred

2- The peritoneal cell population is extremely stable after they reach 180gm body weight, and remains so until at least two years of age. Also, there are no differences in cell populations that exist between males and females (Mackenzie et al.1981).

3- Pilot studies with chitosan indicated that responses in the peritoneal cavity of these rats strains paralleled that seen in the skin.

The approval for animal use was provided by the Institutional Animal Care and Use Committee of Western Michigan University, Kalamazoo (Approval No. LACUC protocol NO.03-06-01, and LACUC protocol NO. 06-06-01). The rats were kept at the laboratory animal house, Western Michigan University, on 12-hour light/dark cycle and given water and Purina Rodent Chow food ad libitum. Both males and females were included in the study. The age of the rats at the beginning of the study ranged from 5-14 months. A total of 32 rats were included in the study. All the rats included in the study appeared to be in good health and their average weight was 200 g.

Study plan:

The study period was approximately 2 weeks divided to four time points: 4 hours, 4 days, 7 days, and 15 days. 85% deacetylated chitosan (MW, 600.000 Daltons) sponge-like 1 cm x 1 cm x 0.4 cm pads, (Vanson Halosource Company) was used throughout the study. Gelfoam® (Pfizer Corporation) pads of a similar size were used as the control material for the study. Four replicates for each time point were used for both chitosan and control groups.

Surgery:

Rats were anesthetized with Halothane® (approximately 2.5-3.0%), abdominal hair shaved and the skin sterilized, and incisions made at about 1 cm from the ventral midline. Chitosan pads were placed through the incision site onto the omentum adjacent to spleen, and the abdominal incisions were closed using standard suture material. The rats were sacrificed at 4 hours, 4 days, 7 days, and 15 days. The pads and the attached mesentery and peritoneal tissues were taken and fixed in 10% phosphate-buffered formalin. In addition, the spleen, mesenteric lymph node, thymus and the pancreas were taken in many animals.

Histotechnology:

Sectioning:

Tissues were fixed in 10% phosphate-buffered formalin, then processed on a vacuum infiltration tissue processor (Thermo Electron Excellsior), passed

through a graded series of ethanol; 75% for 5 hours , 90% for 30 minutes , 95% for 30 minutes, 100% for 30 minutes and another two times in 100% for one hour each. Then they were passed two times through xylene for 30 minutes each and one time for 1 hour successively. Following clearing by xylene, the tissues were infiltrated with paraffin three times; twice for 30 minutes each and one time for 1 hour. Afterward, the tissues were embedded in paraffin blocks on a Tissue Tek II (Cryoconsole scientific model 4585, Sakura USA), and then sectioned on a rotary microtome at 5 microns (Reichert Jung, Model 2030). The sections were floated on 37°C – 39°C waterbath (Tissue floating bath Model 135) and dried in a warm oven at 56°C - 59°C for 1-24 hours.

Staining:

Hematoxylin and Eosin was used to stain the majority of sections (Appendix A) . The sections were placed two times in Xylene for 5 minutes each followed by two times in 100% ethanol and twice in 95% ethanol. Then, they were rinsed in distilled water and placed in Lerner 2 hematoxylin for one and a half minutes. After that, the sections were dipped 2-3 times in 1% glacial acetic water and then placed into running tap water for 2-3 minutes followed by rinsing in 95% ethanol. Then, they were placed in Eosin-Phloxine for 2 minutes and rinsed in 95% ethanol. Subsequently, the sections were rinsed four times in 100% ethanol, 3 times in Xylene and coverslipped using synthetic mounting media.

Microscopy:

The slides were scanned under 20X power magnification , and analyzed under 40X magnification so as to aid identification of the cells involved in the reaction associated with the chitosan and Gelfoam® pads.

Assessment:

For each section, the pad region and the surrounding host tissue were divided into three zones:

A) The pad zone represented by the region in the center which contains the remaining chitosan pad.

B) The reactive zone is the region of tissues surrounding the pad zone where the cellular reaction to the pad was occurred.

C) The omental zone is the omentum outer layer which surrounds the pad and the reactive zone.

Scoring of the cellular response in the reactive zone:

The diameter of the reactive zone was assessed as a measure of the magnitude of the host reactions towards the chitosan and the Gelfoam® pads. 12 replicate measurements separated by about 20 mm each were counted for each section. These 12 readings were used to calculate and compare the size of the reactive zones. Additionally, from these 12 replicated readings across the reactive zone the following parameters were also measured;

1- The number and the types of the cells present in the reactive zone. Cells were distinguished as either granulocytes, macrophages/ monocytes, giant cells, mast cells, lymphocytes or plasma cells. The average percentage of each cell types was calculated at each time point.

2- Cells were divided to the following groups (according to their morphology);

a) Mononuclear cell type which includes macrophages, lymphocytes and plasma cells.

b) Multinuclear cell type which is mainly consisted of giant cells.

c) Polymorphonuclear cell type consisting of neutrophils , eosinophils and basophils.

d) The fibrocytic cell type composed of fibrocytes and fibroblasts.

3- The changes in cell number and in the number of the vessels in the reactive zone were estimated for each time point.

4- The amount of fibrosis was defined by the amount of collagen present (as defined by the histochemical staining procedure of Masson's Trichome). The area of collagen positivity was measured using Metamorph® Image Analysis program. The area of the pad was subtracted from the area of the collagen histochemical positivity to provide the area of collagen deposition surrounding the pad.

Masson's Trichome Procedure :

The sections were deparaffinzed and hydrated before the Masson's stain was applied (Appendix B). Sections were rinsed in Bouin's solution for one hour at 56^0 C or overnight at room temperature (RT). Next, they were washed in tap water and rinsed in distilled water, followed by staining in Weigert's hematoxylin for 10 minutes. Then rinsed in running tap water for 10 minutes and again in distilled water. Afterwards, these were stained in Biebrich Scarlet- acid Fuchsin solution for 1 minute and then rinsed. The sections were then placed in a phosphmolybdic-phosphotungstic acid solution for 15 minutes, stained with aniline blue for 3 minutes and rinsed in distilled water. Finally, they were placed in 1% glacial acetic water for 3 minutes , rinsed, dehydrated through graded series of alcohol, cleared in several changes of xylene, mounted , and coverslipped.

Data Analysis:

ANOVA statistical test was used to compare differences of the magnitude of reactive zones, as well as the differences in the number of various cell types between chitosan and Gelfoam ® at different time points. The level used to define a significance difference was a p-value of 0.05.

3- Immunohistochemistry:

The specific immune cells infiltrated in the tissues surrounding the chitosan and the Gelfoam® pads were immuophenotyped by using CD3 and

CD20 monoclonal antibodies. The immunhistochemical assay was carried out on formalin-fixed, paraffin embedded tissues. The sections were first deparaffinized in two changes of xylene for 10 minutes each at RT, then rinsed in three changes of 100% ethanol, two changes of 95%, one change of , 80% , 70%, 50% ethanol, and then rinsed several times in distilled water, to be finally placed in Tris buffered saline (TBS) for a minimum of 5 minutes. For antigen retrieval, the slides were treated with heat induced epitope retrieval (HIER) solution (Antigen retrieval CITRA HK086-9K Biogenex, CA). The HIER was dissolved in TRIS/EDTA buffer in coplin jar and preheated to 95%-100% (appendix C), then the sections were placed in the jar, incubated in a rice steamer for 30 minutes and then rinsed in several changes of distilled water. To block the endogenous peroxidase, the sections were placed in 6% hydrogen peroxidase/methanol bath for 30 minutes at RT, rinsed in running tap water for 5 minutes followed by rinsing in several changes of distilled water. The slides were placed in TBS+ Tween 20 (TBT999 ScyTek, Utah) for 5 minutes at RT. The subsequent steps were carried out in Dako automiser universal staining system (software version V3.1.1) with the following scheme .

Table 4 Reaction scheme in the Automiser staining system.

Template step	Staining information
Protein block	Normal horse in NAD 1:28 –30 minutes
Buffer- blow	Blow
Auxiliary reagents	Avidin-15 minutes
Buffer rinse	TBS-T20
Auxiliary reagents	Biotin-15 minutes
Primary antibody	CD3 1:50 in NAD for 60 minutes and for control Normal Horse serum 1:28
Buffer rinse	TBS-T20
Secondary antibody	Horse anti-mouse (rat-absorbed) at 1:100 for 30 minutes
Buffer rinse	TBS-T20
Tertiary reagent	R.T.U. ABC Elite Peroxidase reagent for 30 minutes
Buffer rinse	TBS-T20
Substrate batch	Nova Red for 15 minutes
Distilled water rinse	Distilled water

The sections were incubated in normal horse serum (S-2000 Vector, CA) at 1/28 concentration for 30 minutes, and then rinsed in tap water. 0.01% of Avidin D (A-2000 Vector, CA) solution was applied in the sections for 15 minutes at RT , and rinsed in three changes of TBS+T20. Afterwards, 0.03% of Biotin (B4501 Sigma MO) solution was applied for 15 minutes at RT, and then the sections rinsed with three changes of TBS+T20 . The primary antibody with specificity for CD3 (Pc3/188A, Sc-20047 Santa-Cruz Biotechnology) was diluted in normal antibody diluent (ADT 999, ScyTek, Utah) and used at 1/50 concentration. The same dilution procedure was followed when primary antibody CD20 (M-20, Sc – 77735 Santa-Cruz Biotechnology) was used. The primary antibody was added to the sections for one hour at RT, then sections were rinsed with three changes of TBS+T20 and stayed in the last change for 2

minutes. The secondary antibody, biotinlyated anti-mouse (rat absorbed) (BA-2001, Vector CA) IgG H+ L (made in the horse) was diluted to 1:100 in the normal antibody diluent and applied for 30 minutes at RT, and the sections rinsed in three changes of TBS+T20 . The tertiary reagent, R.T.U. Elite peroxidase (PK-7100, Vector CA) was added and the slides were incubated for 30 minutes at RT, and rinsed with three changes of TBS+T20 and stayed in the last change for 2 minutes . To detect the positive cells, Nova RED (SK-4800, Vector, CA) substrate was applied for 15 min at RT, and then the slides were rinsed in distilled water. After the slides were taken from the automiser system, they were counterstained by Lerner's Haematoxylin for 1½ minute, dipped 2-3 times in 1% aqueous glacial acetic acid , and placed under running tap water for 2 minutes. Finally, they were dehydrated through 2 changes of 95% ethanol, 3 changes of absolute alcohol , cleared in three changes of xylene and mounted.

Controls:

For the negative control, the primary antibody was replaced by the normal horse sera in the assay . For the positive controls; thymic tissue was used as a control for CD3 , and splenic tissue for CD20 antibodies .

Grading system:

The lymphocytes were enumerated and categorized either as positive or negative based upon the binding of the CD3 and CD20 antibody . The result assessed on a grade scale ; level 1 (+) where <20 cells were positively

stained, level 2 (++) where moderate (50-75) cells were stained, and level 3 (+++) was interpreted as high positivity where >100 cells were positive,

CHAPTER 3.

RESULTS

RESULTS

1. Microbiological results:

1.1 Microbroth dilution assay results:

Acid solvent effect:

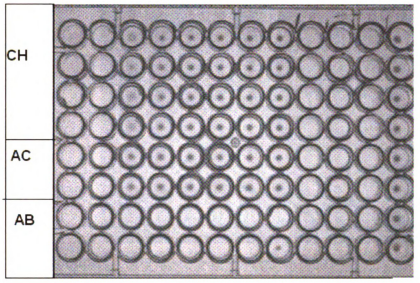
Two organic acids, acetic and formic acid were used as solvents for the test samples. The bactericidal activity of two different forms of chitosan and control substances against *E.coli* ATCC 25922 strain was measured at pH 5.0. No samples inhibit the growth of bacteria with either acid. At 1% acetic concentration, the *E.coli* strain was two- to four -fold more sensitive to MSU 033 (62.5 µg/ml) than to other forms . (Table 5). The MIC of both MSU 035 form and the acetic acid is (250 µg/ml) (Figure 5.1, 5.2 & 5.3). The MIC of Kanamcyin ranged between 0.2-0.4 µg/ml. However, at 0.2M formic acid neither the chitosan forms at any concentration, nor the formic acid inhibit the growth of *E.coli.* (Figure 6.1, 6.2, 6.3,& 6.4).

Table 5. The MIC values of chitosan forms at different acid solvents .

Test samples	Minimum inhibitory concentration (MIC) (µg/ml)	
	Acetic acid	Formic acid
MSU 033	62.5	0
MSU 034	125	0
MSU 035	250	0
Acetic acid	250	NA
Formic acid	NA	0
Kanamycin	0.2-0.4	0.2-0.4

NA= not applicable

Figure 5.1. Bactericidal activity of cellulose sample MSU033 against *E.coli* Strain dissolved in acetic acid



AB= Antibiotic, **AC**= Acid, **CH**= Chitosan

Figure 5.2. Bactericidal activity of chitosan sample MSU034 against *E.coli* strain dissolved in acetic acid

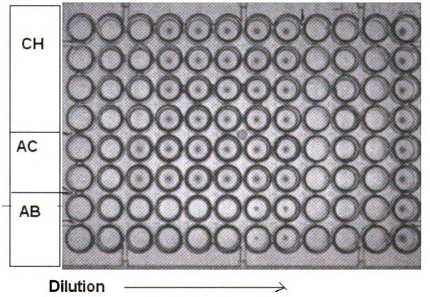
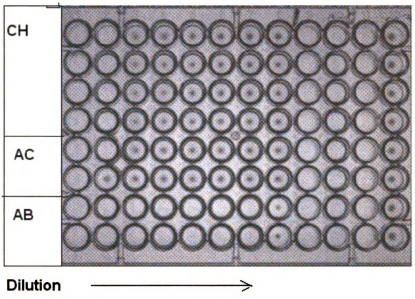


Figure 5 .3. Bactericidal activity of chitosan sample MSU035 against *E.coli* strain dissolved in acetic acid .



AB= Antibiotic, **AC**= Acid, **CH**= Chitosan

Figure 6 .1. Bactericidal activity of cellulose sample MSU030 against *E.coli* strain dissolved in Formic acid

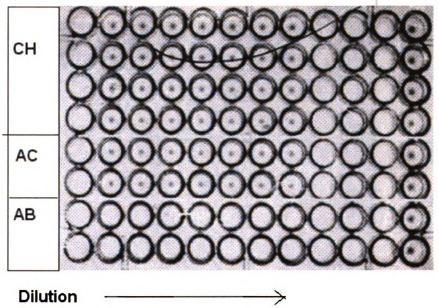
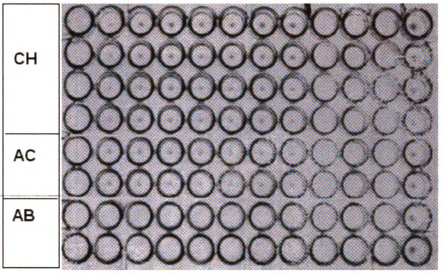


Figure 6.2. Bactericidal activity of cellulose sample MSU033 against *E.coli* strain dissolved in Formic acid .



AB= Antibiotic, **AC**= Acid, **CH**= Chitosan

Figure 6 .3. Bactericidal activity of chitosan sample MSU034 against *E.coli* strain dissolved in Formic acid at.

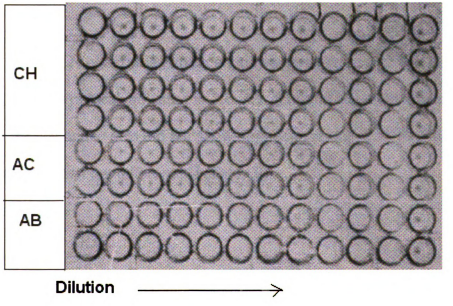
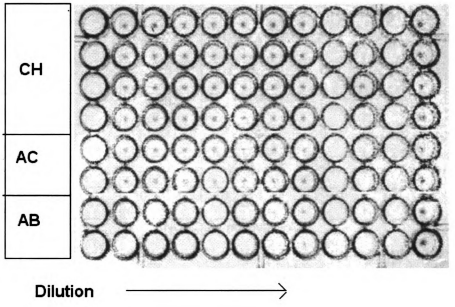


Figure 6.4. Bactericidal activity of chitosan sample MSU035 against *E.coli* strain dissolved in Formic acid .



pH effect:

The pH effect was examined in two concentrations of acetic acid, 1% and 2%, and the pH was adjusted to 2.6, 4.2, 5.0, and 6.0. All forms of chitosan at 1% or 2% acetic acid did not show antibacterial activity. No difference was found between the different pH (Table 6)

Table 6. The MIC values of chitosan forms and controls at various pH.

Test Sample	MIC (µg/ml)			
	1% acetic acid		2% acetic acid	
	pH 2.6	pH 5.0	pH 4.2	pH 6.0
MSU 033	125	62.5	0	0
MSU 034	250	125	0	0
MSU 35	ND	250	0	0
Acetic acid	62.5	250	125	125
Kanamycin	0.2-0.4	0.2-0.4	0.2-0.4	0.2-0.4

ND= Data not available,

Mode of sterilization effect:

No difference was observed In the MIC values between the autoclaved and non-autoclaved test samples solutions at pH 2.6 (Table 7) . The *E.coli* strain was more sensitive to acetic acid than to chitosan .

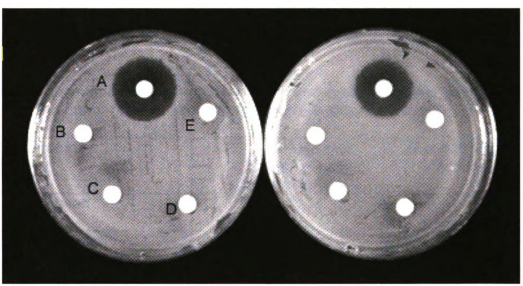
Table 7. Effect of mode of sterilization on the chitosan antibacterial activity.

Test sample	MIC (µg/ml)	
	Autoclaving	No autoclave
MSU 033	125	125
MSU 034	250	250
Acetic acid	62.5	62.5
Kanamycin	0.2-0.4	0.2-0.4

1.2 Agar disk diffusion assay:

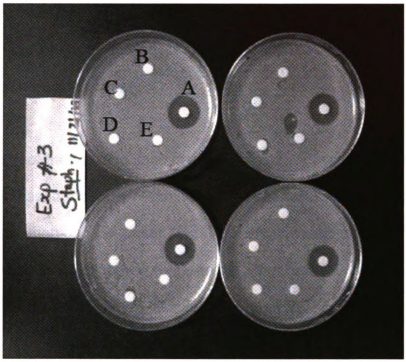
An amphoteric form of chitosan was used in these specific experiments. Paper disks were saturated with different concentrations of chitosan, and placed in agar plates, and the susceptibility of the bacteria to chitosan was determined as either resistant (- ve) or sensitive (+ve). The three different concentrations of chitosan did not inhibit the growth of the two strains of bacteria as no inhibition zone was observed (Figure 7 &8). However, the two strains were sensitive to Kanamycin antibiotics, and the diameter of the inhibition of the growth zone for *S.aureus* was 18mm and for *E.coli* was 20mm (Table 8)

Figure 7. The antibacterial activity of amphoteric chitosan and controls against *E.coli* by agar diffusion test.



A= Kanamycin, B= 0.5% chitosan, C= 0.25 chitosan, D= 1% chitosan, E= Sodium succinate.

Figure 8. The antibacterial activity of amphoteric chitosan and controls against *S.aureus* by agar diffusion test.



A= Kanamycin, B= 0.5% chitosan, C= 0.25 chitosan, D= 1% chitosan, E= Sodium succinate.

Table 8. The antibacterial activity of chitosan and controls against gram- negative and gram -positive bacteria by agar disk diffusion assay.

Test samples	*S. aureus* zone of inhibition (mean diameter in mm)	*E.coli* zone of inhibition
1% chitosan	–*	–
0.5 % chitosan	–	–
0.25 % chitosan	–	–
Sodium succinate	–	–
Kanamycin	+ •(18)	+ (20)

- Negative no zone of inhibition, • positive with zone of inhibition.

2. Cellular response to intra-peritoneal implantation of chitosan:

2.1 Effect of chitosan implantation on the mononuclear and polymorphonuclear cells:

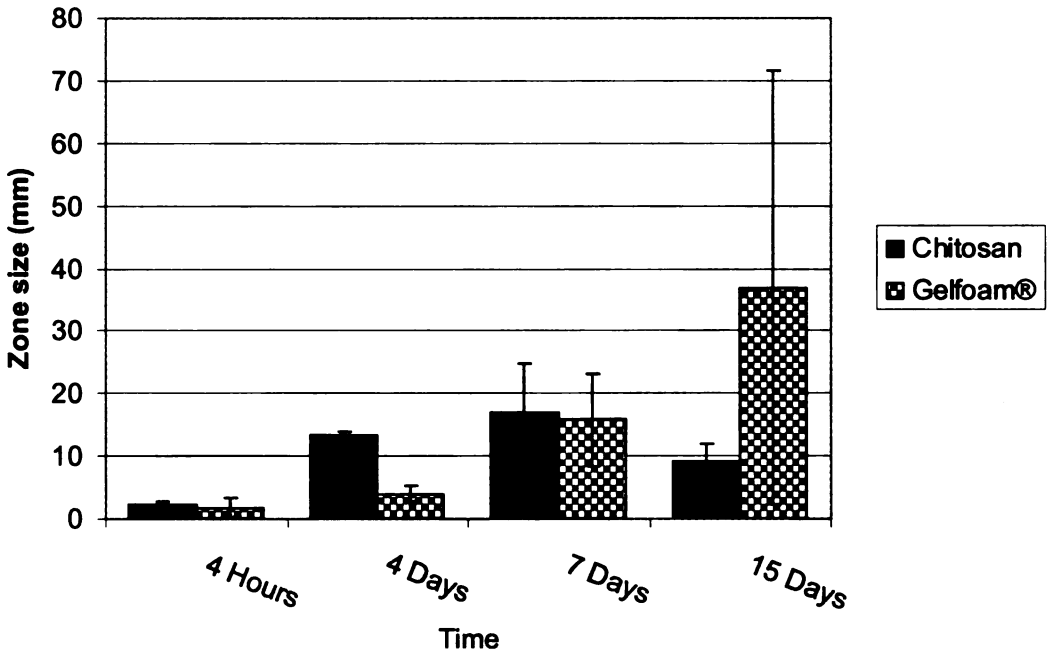
The size of the reactive zone, the area of cellular reaction surrounding the pads, was increased with time reaching its peak at 7 days post-implantation (16.9 mm), and then declining after 15 days in the chitosan group of rats (9.2 mm) (Table 9). In the Gelfoam® group, on the other hand, it continued to increase until day 15 (36.9 mm) (Table 9 & Figure 9).

Table 9. The cell types in the reactive zone after implantation of chitosan and Gelfoam® pads at various time points.

Parameters	4 Hours (X±SD)		4 Days (X±SD)		7 Days (X±SD)		15 Days (X±SD)	
Treatment	CH	GF	CH	GF	CH	GF	CH	GF
Reactive zone size (mm)	2.1 (±0.6)	1.8 (±0.3)	13.2 (±6.2)	4.0 (±1.5)	18.8 (±6.5)	11.8 (±5.4)	9.2 (±2.9)	36.9 (±34.8)
Mononuclear cells (%)	48.7 (±14.1)	63.5 (±19.5)	81.7 (±11.1)	57.3 (±18.1)	67.2 (±4.5)	57.1 (±31.1)	74.6 (±6.4)	82.2 (±7.6)
Polymorpho-nuclear cells (%)	28.4 (±12.2)	6.2 (±4.4)	1.8 (±2.0)	14.5 (±21.1)	6.1 (±5.0)	1.8 (±1.8)	4.5 (±3.6)	0.7 (±0.4)
Mast cells (%)	18.6 (±6.9)	10.0 (±6.2)	0.6 (±0.4)	4.8 (±2.7)	2.4 (±1.0)	3.5 (±0.7)	0.3 (±0.5)	1.6 (±1.4)

CH= Chitosan, GF= Gelfoam

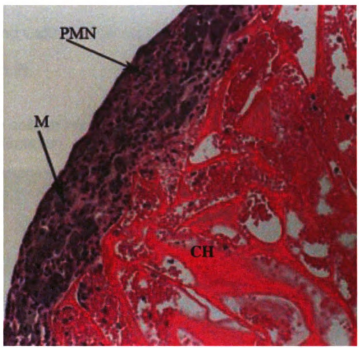
Figure 9. The reactive zone size of chitosan and Gelfoam® at various time point.



The most common cell type found after 4 hours of implantation were mast cells and macrophages. In both the chitosan and Gelfoam® group, there was a diffuse infiltration of inflammatory cells, macrophages and polymorphonuclear cells (PMN), in the reactive area surrounding the pad that started as early as 4 hours post- implantation

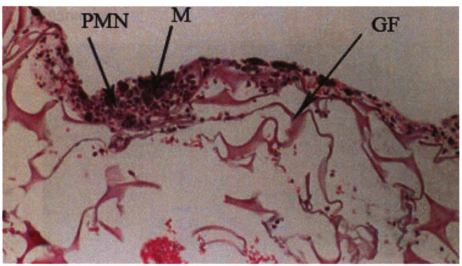
(Figure 10.1 & 10.2).

Figure 10.1. The cellular reaction surrounding the chitosan pad at 4 hours post-implantation.



CH= Chitosan, M= Mast cells, PMN= Polymorphonuclear cells

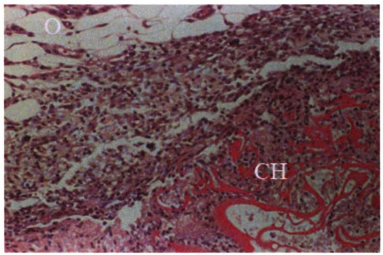
Figure 10.2. The cellular reaction surrounding the Gelfoam® pad at 4 hours post-implantation.



CF= Gelfoam®, M= Mast cells, PMN= Polymorphonuclear cells

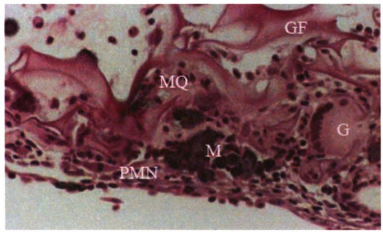
After 4 days of implantation the number of mononuclear cells and fibrocytic cells continued to increase, while the PMN cells were decreased for both chitosan and Gelfoam ® groups (6.2% and 1% respectively) (Table 9 and Figure 11.1 & 11.2).

Figure 11.1 The cellular reaction surrounding the chitosan pad at 4 days post-implantation.



CH=chitosan, O= Omentum

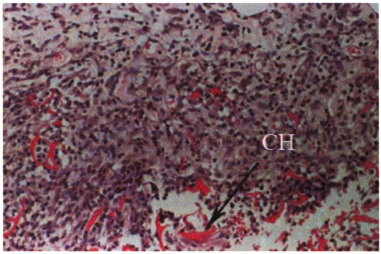
Figure 11.2 The cellular reaction surrounding the Gelfoam® pad at 4 days post-implantation.



G= Giant cells, GF= Gelfoam®, M= Mast cells,
MQ= Macrophages, PMN= Polymorphonuclear cells

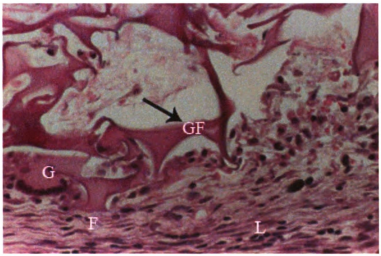
Large numbers of mononuclear cells and low numbers of mast cells and PMN cells were observed at day 7 post- implantation in both chitosan and Gelfoam® groups (Figure 12.1 &12.2).

Figure. 12.1 The cellular reaction surrounding the chitosan pad at 7 days post-implantation



CH= Chitosan

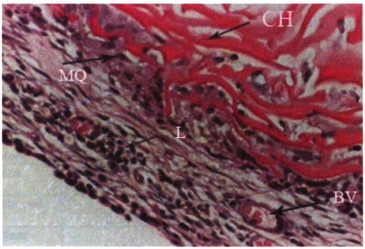
Figure 12.2. The cellular reaction surrounding the Gelfoam® pad at 7 days post-implantation.



F= Fibrocytic cells, G= Giant cells, GF= Gelfoam®, L= Lymphocytes

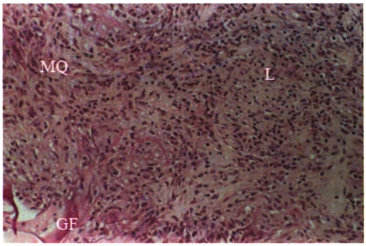
At 15 days post-implantation there was a decrease in the zone size and number of mononuclear cells samples from the chitosan group (9.2 mm and 74.6% respectively) compared to the Gelfoam® group (36.9 mm and 82.2% respectively) (Figure 13.1& 13.2).

Figure. 13.1 The cellular reaction surrounding the chitosan pad at 15 days post-implantation.



BV= blood vessel, CH= chitosan, MQ= Macrophages, L= Lymphocytes

Figure 13.2. The cellular reaction surrounding the Gelfoam® pad at 15 days post-Implantation.



GF= Gelfoam®, MQ= Macrophages, L= Lymphocytes

ANOVA tests showed that the size of the reactive zone of the chitosan group was statistically significant from the zone of the Gelfoam® group (p=<0.05) (Table 10). No significant difference was seen between chitosan and Gelfoam® groups in terms of the inflammatory cells, mononuclear and PMN, (p>0.05) (Table 10). However, the number of PMN cells significantly decreased with time in each group (p=<0.005)

Table 10. ANOVA result of the effect of different treatment on the magnitude of the reaction and the cell types at various time points.

Parameters	Time	Treatment	Time &Treatment
Zone size	0.011*	0.304	0.021*
Mononuclear cell	0.146	0.823	0.136
Multinuclear	0.023*	0.001*	0.045*
Polymorphonuclear cells	0.001*	0.359	0.697
Lymphocytic cells	0.157	0.001*	0.005*
Blood vessels	0.002*	0.213	0.364

Time: 4hrours & 4,7 15 days ;Treatment: Chitosan or Gelfoam; * *p*-value is significant at $p<0.05$

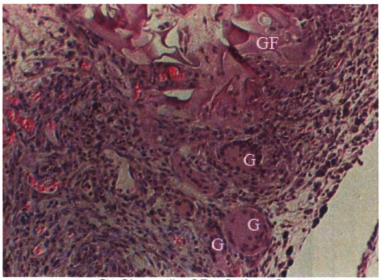
2.2. Effect of chitosan on the multinuclear cells:

The number of multinuclear cells (giant cells) present in the reactive zone increased with time in both the chitosan and control groups (Table 11). However, they were significantly increased in the Gelfoam group ® compared to the chitosan group (p<0.05) (Table 10, Figure 14).

Table 11. The cell types and the blood vessels in the rat tissues after implantation of chitosan and Gelfoam® pads at various time points.

Parameters	4 Hours (X±SD)		4 Days (X±SD)		7 Days (X±SD)		15 Days (X±SD)	
Treatment	CH	GF	CH	GF	CH	GF	CH	GF
Fibrocytic Cells (%)	2.7 (+2.6)	0.2 (+0.4)	10.0 (+6.7)	32.1 (+16.5)	24.2 (+8.6)	31.5 (+38.8)	20.9 (+6.5)	12.5 (+8.2)
Multinuclear Cells (%)	0	0	0.2 (+0.2)	3.4 (+1.4)	0.6 (+0.1)	2.4 (+0.1)	0.1 (+0.1)	2.9 (+2.9)
Indefinable (%)	6.7	5.5 (+ 2.6)	1.5 (+1.1)	1.0 (+ 0.8)	1.9 (+1.1)	0.5 (+ 0.7)	3.0 (+1.5)	0
Blood vessels	0	0	72 (+30.1)	36 (+45)	145 (+55.6)	40 (+6.4)	107 (+28.3)	123 (+54)

Figure 14. Giant cells in the reactive zone in a sample from the Gelfoam® group.

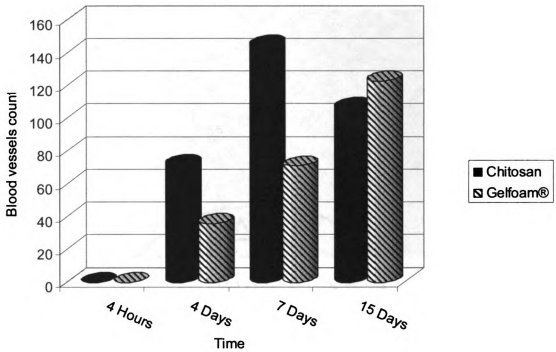


G= Giant cells, GF = Gelfoam®

2.3. Effect of chitosan on the formation of blood vessels:

It appears from the observations of the reactive zone surrounded the pads that chitosan has greater angiogenic activity than Gelfoam ® (Table 11). The number of blood vessels adjacent to pads was greater specifically at day 4 and day 7 post-implantation with chitosan than with Gelfoam ® (Table 10 & 11). However, after 15 days of implantation, the blood vessels numbers decreased in the chitosan group compared to the Gelfoam® group (107/mm and 123/mm respectively). Overall, neovascularization was more prominent in the chitosan group compared to the Gelfoam® group (Figure 15).

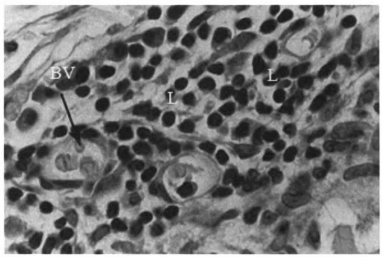
Figure 15. The blood vessels in the reactive zone of chitosan and Gelfoam® group at various time points.



2.4 Effect of chitosan on the lymphocyte cells:

At all points in the time course that were studied, the lymphocytic cells, i.e. lymphocytes and plasma cells, were significantly greater in the tissues with chitosan than with Gelfoam® (Table 10). Starting as early as 4 days post-implantation lymphocytes were present, and they increased with time in the chitosan group and reached a peak at day 15 post-implantation. In contrast, these cells in the Gelfoam® group decreased with time. (Figure 16).

Figure 16. Lymphocytes in the reactive zone in a sample from the chitosan group.



BV= blood vessel, L= Lymphocytes

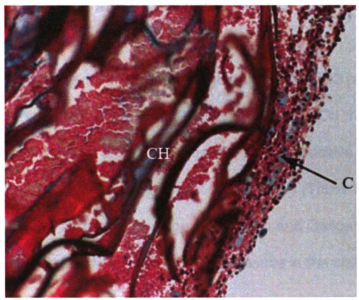
2.5. Effect of chitosan on Collagen deposition:

Collagen presence was used as index of the amount of scar tissues that develops around the pads. Both chitosan and Gelfoam® groups showed increased amounts of collagen until day 7 post-implantation (Figure 17.1 & 17.2). However, at 15 days post-implantation, the two groups showed different patterns of collagen deposition. The amount of collagen was significantly less in the chitosan group (2.51 μm^2), compared to the Gelfoam® group where it increased significantly ($p<0001$) (Table 12).

Table 12. Collagen deposition in the chitosan and Gelfoam® groups at various time points.

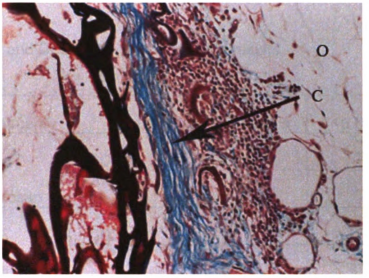
Time	Collagen (μm^2)	
	Chitosan (X±SD)	Gelfoam® (X±SD)
4 Hours	0.6±0.22	0.53±0.15
4 Days	2.75±0.84	2.34±1.31
7 Days	5.77±3.7	2.06±1.14
15 Days	2.51±0.29	7.2±4.05

Figure 17.1. Reaction around chitosan after 4 days of implantation. Minimal collagen deposition with Masson's Trichome stain.



C= Collagen, CH= Chitosan

Figure 17.2. Reaction around chitosan after 15 days of implantation. High collagen deposition with Masson's Trichome stain



O= Omentum, C = Collagen

3. Immunohistochemistry findings:

Characterization of T cells and B cells using anti-CD3 and anti- CD20 antibodies:

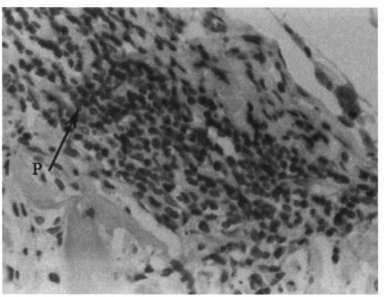
The chitosan group of rats, showed high number of CD3 positive cells at day 4 and 7 post-implantation, and this declined by day 15. On the other hand, the Gelfoam® group showed a moderate number of CD3 positive cells at day 4 and 7, which then increased by day 15 post-implantation (Table 13, Figure 18.1, 18.2 & 18.3). Also, the pattern of reaction of chitosan and Gelfoam® groups was different when CD20 antibodies were used. The tissues in the chitosan group had high counts of positive cells (level 3, +++) at day 7, which then slightly decreased to level 2 (++) at day 15 (Figure 19.1, 19.2, & 19.3). However, low

numbers of CD20 positive cells were found in the Gelfoam® group at day 4 and 7 level 1(+), and these moderately increased by day 15 (Table 13)

Table 13. CD3 and CD20 immunophenotyping results.

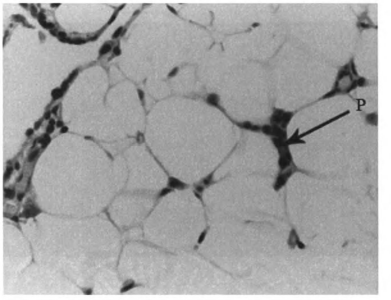
Treatment		4 Days	7 Days	15 Days
Chitosan	CD3	+++	+++	++
	CD20	++	+++	++
Gelfoam	CD3	++	++	+++
	CD20	+	+	++

Figure 18.1. Immunohistochemistry : Example of level 1 (Low) CD3 positivity in chitosan group.



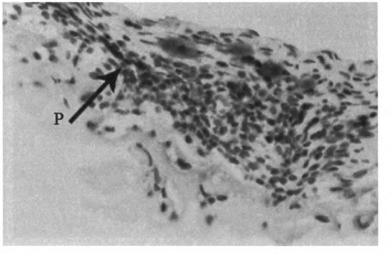
P= positive

Figure 18.2 Immunohistochemistry : Example of level 2 (Intermediate) CD3 positivity in chitosan group



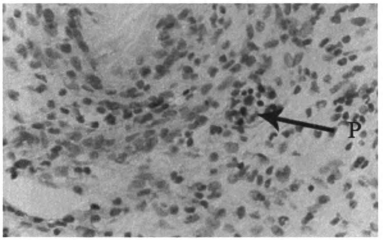
P= positive

Figure 18.3 Immunohistochemistry : Example of level 3 (High) CD3 positivity in chitosan group



P= positive

Figure 19.1 Immunohistochemistry: Example of level 1 (Low) CD20 positivity in chitosan group.



P= positive

Figure 19.2. Immunohistochemistry: Example of level 2 (Intermediate) CD20 positivity in chitosan group

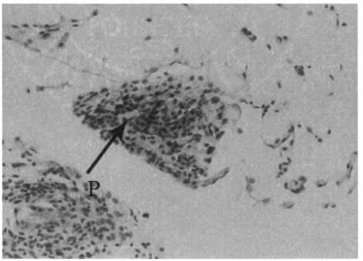
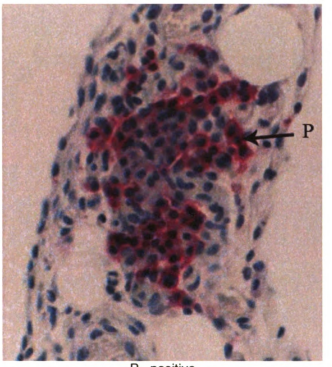


Figure 19.3 Immunohistochemistry: Example of level 3(High) CD20 positivity in chitosan group



P= positive

CHAPTER 4.

DISCUSSION

DISCUSSION

The antibacterial activity of chitosan:

The number of practical applications of chitin and chitosan in the medical field has grown rapidly in recent years. Chitosan, a biodegradable, nontoxic and biocompatible polymer, has been used as an antimicrobial compound in agriculture, and as an agglutinating and flocculating agent in wastewater treatment, as well as a pharmaceutical agent in biomedicine. In this present study, two forms of chitosan were evaluated for their role in wound healing. Two aspects were studied in exploring the compounds role in wound healing. First we explored chitosan's capability to inhibit the growth and penetration of bacteria *in vitro*, because the presence of micro-organisms greatly inhibits wound healing. Second, the role of chitosan in stimulating the cellular components of healing, particularly those associated with immunological responses, were also studied in an intra-peritoneal rat model.

Through testing using both agar diffusion tests and microbroth dilution assays, Two forms of chitosan were found to lack any bactericidal effect against two strains of bacteria; *E.coli* ATCC25922 and *S.aureus* ATCC 29213 . In contrast, many reports have indicated that chitosan has antimicrobial activity against several microorganisms including fungi, algae and different strains of bacteria (Rabea et al. 2003, Taha et al 2002, and Tsai et al 2002). In the first

phase of study a microbroth dilution assay was used and the MIC of each chitosan form was calculated against an *E.coli* bacterial strain. At pH 5.0, the MIC values for MSU034 chitosan was 125 µg/ml, and for MSU035 chitosan was 250 µg/ml. However, the MIC values for the controls, cellulose and the acetic acid, were within the same range as the chitosan samples. The MICs for cellulose samples were 62.5 and 125 µg/ml, and for the acetic acid they ranged from 62.5 to 250 µg/ml. These results are in concordance with a study carried out by Je et al. (2006), where they found that the MIC value of aminoethyl-chitin (AEC) is 62.5 µg/ml, 125 µ/ml for AEC90, and 250 µg/ml for dimethylaminoethyl-chitin (DMAEC 90) when tested against the same bacteria. It could be interpreted that as chitosan produced an MIC value similar to that of acetic acid, the bactericidal effect of chitosan previousely observed may have been entirely due to the presence of the acetic acid alone. The effect of acetic acid was demonstrated by Jia et al (2001) who reported that the antibacterial activity of quaternary ammonium chitosan in acetic acid medium is stronger than this same compound in water, and also that the effects increased as the concentration of the acetic acid increased .

Previous reports showed that the form of acid used as the solvent has an effect on bactericidal activity of chitosan. This study showed that in experiments where formic acid was used as the solvent at pH 5.0, there was no bactericidal effect. This is contrary to the results reported by Chung et al. (2003) who concluded that formic acid without chitosan had best antibacterial effect when

compared to other organic and inorganic acids. Nevertheless, in these experiments the addition of chitosan to the acid greatly enhanced the bactericidal activity. In general, organic acids solvents with lower carbon contents had higher antibacterial effects (Chung et al. 2003).

The amphoteric form of chitosan also did not inhibit the growth of *E.coli* and *S.aureus* bacterial strains when it was used in the agar gel diffusion assay. Loke et al (2000) had a similar experience and did not observe growth inhibition zones against *Pseudomonas aeroginosa* and *S.aureus* strains when chitosan acetate dressing was used. However, zones of growth inhibition were detected when the chitosan acetate dressing was impregnated with Chlorhexidine gluconate compound. Probably, either the form of chitosan used in our experiments contained some acetate salt, which may explain the lack of bactericidal activity, or that chitosan may need to be incorporated with additional antimicrobial substances to improve its antibacterial activity.

The physiochemical, and the consequent biological characteristics of chitosan, vary with the degree of acetylation, the source of chitin, and the overall molecular weight (Rhazi et al 2000). The chitosan forms used in this study have different degrees of acetylation and molecular weight; this fact may explain their weak bactericidal effects. Furthermore, the antimicrobial action is affected by the degree of polymerization, the host, the natural nutrient consistency, the chemical or nutrient constituents of substrates, the environmental conditions, the pH of the growth medium, and the presence or absence of interfering substances such as lipids or proteins (Rabea et al, 2003). Sudarshan et al (1992) showed that

chitosan is no more actively bactericidal at pH 7 due to presence of more uncharged amino groups and poor solubility. Chitosan with MW of 10,000-100,000 has a greater bactericidal action than chitosan of low MW (Rabea et al 2003), though, the chitosan form used in the microbroth assay in our studies has a MW of 97,000 Daltons.

In addition, the antibacterial activity increases with the ionic strength, but is inversely proportional to the pH and metal ion concentration (Chung et al. 2003). It is possible that chitosan compounds used in the present study contains EDTA; Chung et al. (2003) has suggested that the antibacterial activity of chitosan against S. aureus decreases with the addition of EDTA and metal ions . It is possible that the media used in this study, Muellor Hinton broth, contains some metal ions or other inhibitory ingredients.

In all likelihood, there are a number of different mechanisms by which chitosan kills microorganisms and inhibits infections. One of these mechanisms, involves the fact that chitosan is a positively charged molecule; this allows the compound to interact with the negatively charged macromolecules on the surface of the microorganisms (Liu et al 2004). These interactions of the different charged molecules leads to the increase in the permeability of the outer and inner membranes of bacteria, thus resulting in the disruption of membrane integrity and the release of the contents of the cells (Hui et al 2004) . Moreover, chitosan may also bind the DNA in the nuclei and consequently inhibit RNA synthesis (Hadwiger et al 1986). Lastly, as discussed above, chitosan can act as

a chelating agent and will binds trace metals like Mg, that are essential to the growth of bacteria (Cuero et al. 1991).

The cellular response to intra-peritoneal implantation to chitosan:

In the second phase of the study, the cellular responses towards the intra-peritoneal chitosan implants, including those reflecting activation of an immune response, were correlated with the rate of the wound healing. Wound healing is a dynamic, complex process requiring the integration of many different cell types controlled by a variety of cell growth factors and soluble mediators (Singer and Clark 1999). Healing consists of three main overlapping phases; inflammation, tissue formation and tissue remodeling. Chitin and chitosan are both thought to have stimulatory effects on the tissue reactions involved in these three phases of healing.

Several mechanisms may be in play when chitosan and chitin accelerate the healing of wounds. In the present study, the zone containing reactive cells surrounding the chitosan pad was found to be statistically significantly different from that surrounding the Gelfoam® pads in a number of ways. Cells were seen in this location as early as 4 hours post-implantation of the chitosan with the cell number in this zone decreased with time. In contrast, the number of cells in the zone area surrounding the Gelfoam® pads continued to increase until day 15 post–implantation. A significant decrease in the capsule thickness surrounding chitosan was seen with time in our study as was seen in earlier studies (VandeVord et al. 2001). The most common cells accumulate within the reactive zone of chitosan and Gelfoam® groups were polymorphonuclear (PMN),

macrophage and mast cells. After 4 hours of implantation, the percentage of PMN was estimated to be 28.4% for the chitosan group and 18.5% for the Gelfoam® group. However, mononuclear cells, consisting mainly of macrophages were the most dominant cell type, the percentages being 48.7% for the chitosan group and 63.5% for the Gelfoam® group. This finding was similar to Ueno et al (1999) who observed higher counts of PMN and macrophages in chitosan treated beagles 3 days post-wounding compared to non-treated controls. They also observed that at day 6 the inflammation become milder in chitosan treated animals. Macrophage activation was also reported by Pelsuo et al (1994) who stated that chitosan activates macrophages to produce more nitrate, and that it has chemotactic properties to the macrophages. Macrophage activation is essential for wound healing, and results in increases in the metabolic activity, and the secretion of transforming growth factors (TGF-β), platelet derived growth factor (PDGF), and other cytokines (Mori et al 1997, Nishimura 1986).

The inflammatory reaction induced by chitosan varies with the degree of deacetylation (DDA). The DDA of chitosan used in this study was 85%. Hidaka et al. (1999) recounted that chitosan with a DDA ranging from 65% to 80% provokes swelling, severe inflammation and marked neutrophils infiltration which diminished in 2 weeks; whilst the reaction to the chitosan with 94% DDA was only mild and was characterized by fibrosis. These inflammatory cells which are stimulated by chitosan application are critical in wound healing since they are capable of production and secretion of a large range of proinflammatory

cytokines, such as tumor necrosis factor (TNF-α), IL-1, IL-8 and other growth factors, at early stages of healing (Ueno et al 2001).

Various theories has been postulated for the mechanisms involved in the stimulation of inflammation , the enhancement of leukocyte migration, and the accumulation of neutrophils and lymphocytes caused by chitosan. The triggering of inflammation may be due to the activation of Hageman factor and other plasma proteins by the negative charge of the chitosan acetyl group, which elicits blood coagulation, kinin and plasmin production (Hidaka et al. 1999). In the same way, Suzuki et al. (2000) found that chitosan activates complement and induces the release of arachidonic acid products and increase the plasma C3 concentration. The migration of neutrophils may be due to the interaction of chitosan with neutrophil receptors such as selectins (VandeVord et al. 2001). Also, Mori et al (1997) reported that chitosan elicited release of IL-8 from dermal fibroblasts, a factor that is a potent chemokine activator for neutrophils.

Another feature observed in this study was the augmentation of neovascularization by chitosan. More angiogenic activity was associated with chitosan compared to Gelfoam®, specifically at day 7 post-implantation where about 145 blood vessels/mm were counted. Overall, more blood vessels and new vessels were found in reactive zone surrounding the chitosan pads. This result is in concordance with the results reported by Ueno et al. (1999) and VandeVord et al. (2001). A remarkable neovascuolarization was observed in upper layer of wound at day 6 by Ueno et al. (1999). VandeVord et al. (2001) observed an increased angiogenic action associated with the chitosan scaffold that reached

about 4.3 capillaries /mm^2 in week 8. Azad et al 2003 stated that the inducement of angiogenesis by chitosan is attributed to the fact that chitosan speeds up the formation of fibroblasts at early stages of healing. Neovascularization supplies the wound with oxygen, and white blood cells, all of which accelerate wound healing, and defend wounds from infection (Kjolseth et al.1994).

In the present study, the number of multinuclear cells (giant cells), present in the reactive zones surrounding the chitosan pad was found to be significantly less than in the zones surrounding the Gelfoam® pad ($p<0.05$). This may enhance the potential of chitosan in wound healing, as excessive granulation would cause delay in epithelization and result in delay of wound healing (Johnson 1990)

Another important aspect this study demonstrates was the significant difference in the collagen deposition between samples of chitosan and the control group. The amount of collagen was increased with time in both treated rat groups that reached its peak (5.77 μm^2) at day 7 in the chitosan group. It then declined by 15 post-implantation in the chitosan group, though, it continued to increase in the Gelfoam® group (7.2 μm^2). A similar result was observed in a parallel study by our lab in the punch biopsy wounds in rat's dorsal skin (Mackenzie et al 2006). By using Immunohistochemical staining, Ueno et al (1999) also noticed high amounts of collagen and more granulation tissues at day 9 post-wounding in the chitosan group. However, in their study the thickness of the granulation tissue of the chitosan group was continued to increase more than in the control group until day 15. Our results are in agreement with Azad et

al (2003) who observed a higher degree of epithelization and a lack of scarring when chitosan mesh was applied on patients wounds compared to Bactigras bandages. Our result was at odds with Kojima (2004) who found collagen synthesis increased up to day 14.

The decrease in the collagen deposition associated with chitosan could be explained by the action of chitosan in inhibiting fibroblast-mediated contraction of collagen lattices and so reduce contraction of the wound and scarring (Howling et al. 2002). Similarly, Taravel and Domard (1995) observed that when chitosan is in excess it can denature collagen.

Lastly, the immunological nature of the cellular response was investigated by determining the presence of particular lymphocyte cell types involved in the immune complex – T and B cells using two antibodies, CD3 and CD20 respectively. The CD3 immunostain revealed that more T-cells were associated with chitosan pads (>100) at day 4 and 7 post-implantation compared to the Gelfoam® pad. However, at day 15 CD3 positive cells declined in chitosan group and increased in the control group. On the hand, B- cells detected by CD20 immunostaining were about (50-75 positive cells) in samples from both chitosan and Gelfoam® groups at day 15 post-implantation. However, in this study when the plasma cells and lymphocytes were counted in the H+E sections, the chitosan group samples showed an increase of these cell types with time, and in the Gelfoam® group they decreased. Chitosan was reported to have immunomodulatory action and effects as an immune adjuvant (Nishimura 1985, Seferian and Martinez 2001). Obminska-Mrukowicz et al (2006) reported that

administration of chitosan adipate increases the CD4+ and CD19+ cells but not the CD3+ in the mesenteric lymph nodes. Contrary to our results VandeVord et al. (2001) detected low to negative cellular or antibody response in their study to assess the biocompatibility of chitosan. They interpreted their findings to show that chitosan had chemotactic effect on the immune cells but did not lead to a specific humoral immune response (VandeVord et al.2001).

Conclusion:

This study supported the concept that chitosan contributes positively to the healing of wounds. So far, the two forms of chitosan used in this study did not exhibit bactericidal activity against *E.coli* and *S. aureus* strains. Their MIC values were similar to controls, cellulose and acetic acid. Chitosan implanted in the peritoneal cavity induced a significant cellular response with recruitment of inflammatory cells as well as other immune cells to the wound at an early stage post-wounding. However, throughout the 15 day period of the study, the cellular response decreased and became milder in comparison to the Gelfoam®. Another important feature associated with chitosan treatment, is the minimal deposition of collagen and subsequent reduced scarring. Lastly, the use of CD3 and CD20 antibody markers indicated that chitosan stimulates the rapid +accumulation of T cells and B cells in the responding tissue reaction.

APPENDICES

Appendix A
HEMATOXYLIN & Eosin

Lerner 2 hematoxylin :

Eosin Phyloxine:

95% Ethyl alcohol	800 ml
1% Eosin Y (aqueous)	100 ml
Phloxine b (1% aqueous)	10 ml
Glacial acetic acid	4 ml

1% glacial acetic water:

glacial acetic acid (concentrated)	9 ml
Distilled water	99.1 ml

Appendix B

MASSON'S TRICHOME CHEMICALS

Bouin's solution:	
Picric acid	750.0 ml
37%-40% Formalin	250 ML
Glacial acetic acid	50 ml
Weigert's Hematoxylin stock –solution A	
Hematoxylin	1gm
95% Ethyl alcohol	100 ml
29% Ferric Chloride:	
Ferric chloride lumps	29 gm
Distilled water	100 ml
Weigert's Hematoxylin stock –solution B	
29% Ferric Chloride	4 ml
Distilled water	95 ml
HCL , concentrated	1 ml
Weigert's Hematoxylin working solution :	
Weigert's Hematoxylin stock –solution A	20 ml
Weigert's Hematoxylin stock –solution B	20 ml
Filter before use	
1% Aqueous Biebrich Scarlet stock solution:	
Biebrich Scarlet	1gm
Distilled water	100 ml
1% Aqueous acid Fuchsin stock solution:	
Acid Fuchsin	1 ml
Distilled water	100 ml
Biebrich Scarlet- acid Fuchsin working solution:	
1% Aqueous Biebrich Scarlet stock solution	45 ml
1% Aqueous acid Fuchsin stock solution	5 ml

Glacial acetic acid, concentrated	1 ml

Phosphmolybdic-phosphotungstic acid working solution:

Phosphmolybdic acid	5 gm
Phosphotungstic acid	5 gm
Distilled water	200 ml

Aniline blue:

Aniline blue	2.5 gm
Distilled water	100 ml
Glacial acetic acid, concentrated	2 ml

Appendix C
Immunohistochemistry reagents

Tris HCL 0.1 M, PH 7.6:	
Tris HCL (Sigma No. T4253)	7.45
Distilled water 1L	
Adjust PH to 7.6	
Hydrogen Peroxide/methanol	
30% hydrogen peroxide	4 ml
distilled water	16 ml
diethyl alcohol	80 ml
HIER (working) CITRA method:	
CITRA retrieval concentrates	1 part
Distilled water	9 parts

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