THE EFFECT OF CHITOSAN ON GUIDED BONE REGENERATION: A PILOT STUDY IN THE RABBIT

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Abstract

The purpose of this pilot study was to evaluate the osteogenic enhancing potential of chitosan using a guided bone regeneration model designed for this study. Five adult female New Zealand White rabbits weighing 4.5-5.5kg were used. A commercially pure, threaded-type (3.25mm x 7mm) titanium implant was placed 2-3 threads above bone in each distal femur to create a potential space for bone formation. A titanium reinforced Gore-tex™ barrier membrane was positioned over the implant and secured with the cover screw and a micro-tacking system. For the experimental sites, the distal aspect of the femur was surgically exposed and prepared to receive an implant, barrier membrane and chitosan solution (2mg/ml 2% acetic acid). For the control sites, the contralateral femur was similarly exposed and prepared to receive an implant and barrier membrane without chitosan, allowing individual rabbits to serve as their own controls. Two months after surgery, the animals were sacrificed and block sections of the femurs including the implant, barrier membrane and bone growth area were harvested. Specimens were prepared for non-decalcified ground sections and subjected to microscopic examination. Clinical evaluation of control sites showed typical firm red tissue under the barrier membrane with evidence of early calcification. Clinical evaluation of the experimental sites showed typical firm red tissue under the barrier membrane with a greater amount early calcification as well as evidence of significant bone formation over the barrier membrane. Examination by light microscopy of cross-sections revealed new bone formation over as well as under barrier membranes in both chitosan treated and control sites. Increased bone growth found over barrier membranes in the experimental sites is consistent with previous in vitro findings of enhanced bone formation with chitosan treatment. The findings from this pilot study lend support to the concept of using chitosan as an adjunct to guided bone regeneration.

Keywords: Chitosan, guided bone regeneration, implant, barrier membrane, rabbit model.

Introduction

Bone maintenance and regeneration have become essential concepts in the treatment of periodontal defects, tooth extractions and dental implants. Today, great efforts are being made not only to maintain and prevent bone loss but also to augment and regenerate bone. "Osseointegration" is a term first used by Bränemark to describe the firm anchorage achieved between bone and commercially pure titanium implants without an intervening soft tissue layer. (1) This phenomenon, not seen with previous implant materials, is responsible for the success of dental implants today. Regeneration of resorbed or lost alveolar bone is essential for implant placement and success.

Clinically, the concept of guided tissue regeneration has provided the strongest evidence that osseous healing or regeneration can be enhanced by exogenously applied therapy. At the cellular level, the existence of osteoprogenitor cells with the capacity to produce bone in the wound site and the potential to influence the behavior of these cells offers the opportunity to further enhance regenerative wound healing. Chemical mediators or substances that enhance bone formation are thought to be conducive to periodontal, peri-implant and alveolar ridge bone regeneration. The influence of chitosan, a carbohydrate biopolymer, on bone regeneration is of particular interest. Experiments using chitosan in various animal models have demonstrated its ability to improve hemostasis, decrease fibroplasia and enhance wound healing. (2-11) A variety of in vivo applications by different investigators have also suggested that chitosan may enhance the formation of bone. (12-16)

Although its mechanism of action is not known, chitosan is thought to play a role in cellular migration and tissue organization during the wound healing process. Our previous work showed that chitosan promoted the differentiation of mesenchymal stem cells into osteoblasts and facilitated the formation of bone *in vitro*. (17) No previous studies have attempted to evaluate the potential of chitosan to enhance guided bone formation around titanium implants. The purpose of this (*in vivo*) pilot study was to evaluate the effect of chitosan on guided bone regeneration around a commercially pure titanium implant that was intentionally placed above the crest of bone.

Materials and methods

This study utilized five adult female New Zealand White rabbits weighing approximately 4.5-5.5 kilograms each. The rabbits were anesthetized with a combination of ketamine and xylazine IV followed by continuous inhalation delivery of 1-2% Halothane for the surgical procedure. The legs were shaved,

washed and decontaminated with Betadine® prior to surgical draping. The distal aspect of each femur was surgically exposed via skin incision, muscle dissection and elevation of the periosteum using sterile surgical techniques. The flat surface on the lateral aspect of the distal femur was selected for implant placement and guided bone regeneration. The implant site was drilled in the usual manner with profuse irrigation. A commercially pure, threaded-type (3.25 mm x 7.0 mm), titanium implant (Implant Innovations, Inc., Palm Beach Gardens, FL) was placed approximately 2-3 threads above bone. (Figure 1) A titanium reinforced Goretex™ barrier membrane (WL Gore, Flagstaff, AZ) was positioned over the implant to create a potential space (Figure 2) for guided bone growth and secured with the coverscrew in the center and a micro-tacking system at the periphery. (Figure 3)

The experimental sites received one milliliter chitosan solution (2 mg/ml 2% acetic acid). Each experimental membrane was soaked in chitosan solution and allowed to dry in a laminar flow hood. Control sites were treated in the same manner without chitosan. Baseline radiographs were taken and surgical sites were closed in layers. Muscle, fascia and internal dermal layers were sutured with Dexon® resorbable sutures while the outer dermis was sutured to primary closure with nylon sutures. Each animal recovered without complications and received 0.25 g Cefazolin IM per day for three days. Animals were free to move around the cage, had water ad libitum and were fed rabbit chow.

Following two months of healing, animals were sacrificed with 1.0 cc Euthanasia-6 IV and wound sites were surgically exposed and clinically examined. Clinical photographs and radiographs were taken. Block sections of each distal femur including the implant, barrier membrane and the regenerated tissue were harvested, fixed in 10% formalin and prepared for non-decalcified ground sections. Histologic cross-sections of tissue growth and implant-bone interface were stained with Masson Trichrome Stain and evaluated by light microscopy.

Results and discussion

Clinical evaluation of control sites showed minimal hard tissue growth over the borders of the barrier membrane. (Figure 4) Under the barrier membrane, these sites showed typical firm red tissue with hard (early calcification) tissue around the borders. Radiographic evaluation of control sites showed the implant integrated in a supracrestal position. Tissues formed under the membrane were not radiographically evident at two months post-operatively suggesting that they were not substantially calcified. Light microscopic examination of cross-sections revealed a supracrestally positioned implant that was osseointegrated

primarily by cortical bone with new bone deposition superiorly and apically from the cortical level along the implant surface. A limited quantity of new bone deposition was observed in the space under and over the barrier membrane.

Clinical evaluation of the experimental sites showed increased hard tissue growth over the borders of the barrier membrane. (Figure 5) Experimental sites displayed ridges of hard tissue formation, contiguous with adjacent bone, covering the barrier membrane. (Figure 6) Chitosan sites showed firm red tissue under the barrier membrane and a greater amount of hard (early calcification) tissue around the borders than controls. (Figure 7) Radiographic evaluation of the experimental sites at two months post-operatively showed the implant integrated in a supracrestal position. Tissues formed under the barrier membrane were not radiographically evident. Light microscopic examination of cross-sections revealed a supracrestally positioned implant that was osseointegrated primarily by cortical bone with new bone deposition superiorly and apically from the cortical level along the implant surface. New bone deposition was observed in the space under as well as over the barrier membrane. (Figure 8: A) Potential space under the membrane with bone regeneration, B) Expanded PTFE barrier membrane, C) Bone formation outside of the barrier membrane.)

Quantitative analysis of differences between the amount of new bone deposition in chitosan treated versus control sites were not determined in this pilot study due to the limited number of specimens examined.

In this study, the chitosan treated sites grossly displayed increased hard tissue growth along the periphery as well as above the barrier membrane as compared to control sites. Clinical experience with guided tissue regeneration has shown that occasional bone growth over the borders of a well-adapted barrier membrane is possible and attributed to the osteogenic potential of the periosteum. The hard tissue growth observed over the barrier membranes of the chitosan treated sites in this study was more substantial than that seen over controls and appeared to be greater than one would expect from a well adapted barrier membrane.

Radiographic evaluation of the guided bone generation areas after two months healing was not conclusive for calcification of tissue within the created space for chitosan treated nor control sites. Light microscopic examination of cross-sections through the implant and the newly generated tissue revealed comparable amounts of new bone formation along the implant surface, under the barrier membrane as well as over the barrier membrane in both the chitosan treated and the control treated sites.

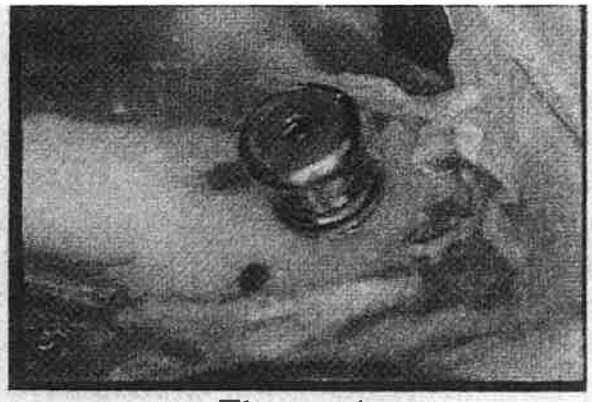
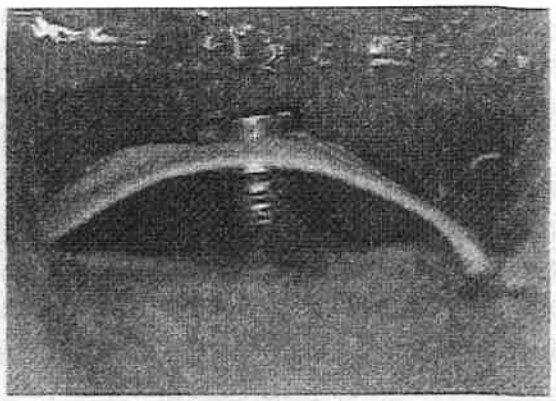


Figure 1



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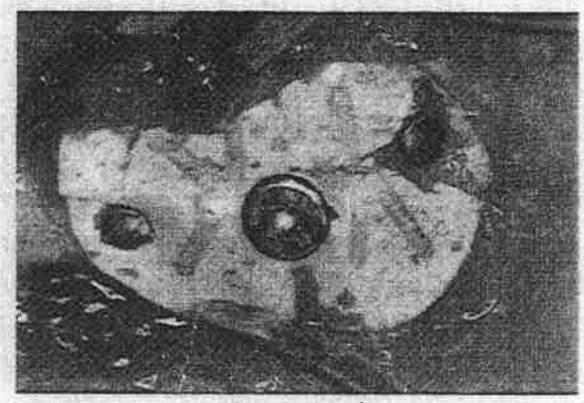

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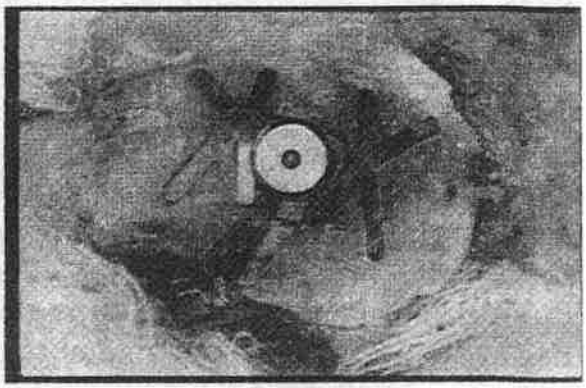

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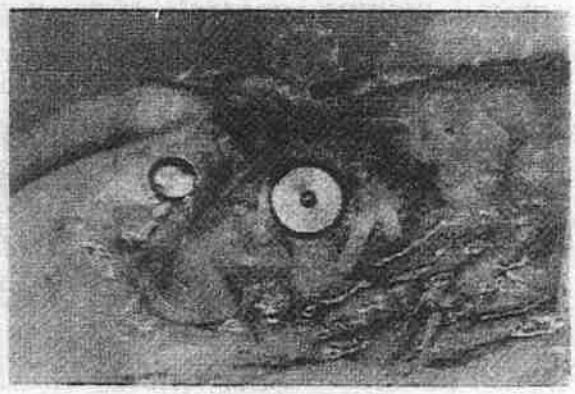

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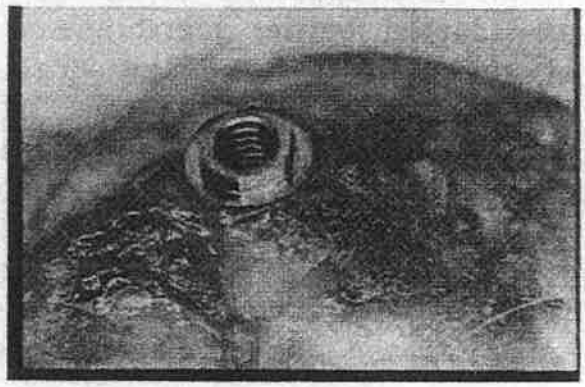

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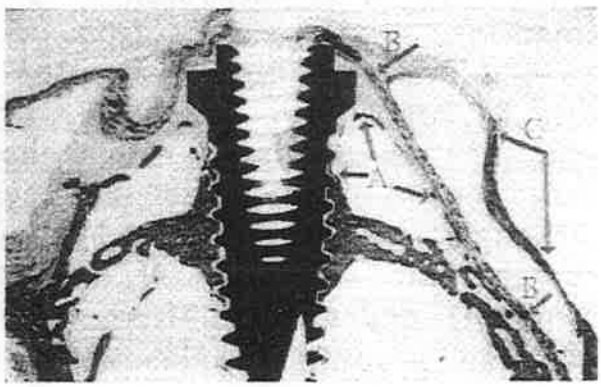

Figure 8

Most of the published reports of chitosan's effect on bone healing are anecdotal. Sapelli, et. al. reported favorable clinical results in case studies using chitosan powder to promote healing of periodontal pockets, palatal wounds and extraction sites. (9) Muzzarelli, et. al. applied chitosan to periodontal wounds and reported decreased fibroplasia with enhanced tissue organization. (11) Subsequently, they applied chitosan to osseous defects (extraction and apicoectomy sites) and concluded, by radiography and biopsy, that it enhanced normal bone formation. (15) Lewis, et. al., reported that chitosan enhanced bone formation in extraction sockets. (12) Unfortunately, these clinical findings are observations from uncontrolled studies with wounds that tend to be self healing. In one controlled animal study, Borah reported that chitosan flake promoted osseous healing of critical size defects as compared to controls in the rat tibia. (13) In a controlled study, Muzzarelli, et. al. evaluated chitosan with imidazole groups in 7mm diameter osseous lesions that were created in the femoral condyle of sheep. The experiemental sites showed signs of neoformed osseous tissue at 20 days and advanced healing at 40 days. Whereas, control sites did not show osseous tissue at 20 nor at 40 days. Control sites were reported to fill with fibrotic reactive tissue or they were void. (16) Our previous *in vitro* work demonstrated that chitosan enhanced the differentiation of mesenchymal stem cells into osteoblasts and facilitated the formation of bone. (17) The findings of the current *in vivo* pilot study are consistent with these published reports and lend support to the concept of using chitosan as an adjunct to guided bone regeneration. Nonetheless, there is a great deal we do not understand and some well designed studies with larger controlled samples are needed to further evaluate chitosan's influence on bone formation.

Chitosan's ability to be made into gels, films, membranes, fibers and beads as well as powders, flakes and solutions has already led to many commercial and biomedical applications (18) and could be advantageously exploited for guided bone regeneration. With its potential to increase bone formation and inhibit fibroplasia, this novel biomolecule may be useful as a biodegradable adjunct to guided bone formation perhaps as a barrier membrane and/or as a carrier for chemical mediators.

Conclusion

The direct observations of hard tissue formation over the barrier membrane in this pilot study suggest that chitosan (solution) may enhance the formation of bone as compared to controls. However, neither radiographic nor histologic examination demonstrated measurable differences in bone formation between chitosan treated sites as compared to controls

under the barrier membrane. Possibly due to the small number of sites tested in this pilot study. The utilization of the rabbit femur appears to be a good model for the evaluation of chitosan (or other chemical modifiers) on the process of guided bone regeneration.

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SAFETY OF PROTASAN™ : ULTRAPURE CHITOSAN SALTS FOR BIOMEDICAL AND PHARMACEUTICAL USE

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Abstract

The use of chitosan in biomedical and pharmaceutical applications is increasing. As such, regulatory requirements concerning the use of chitosan as an excipient, part of a device, or as a drug delivery system will be far more demanding. Chitosan for use in biomedical and pharmaceutical applications will have to be manufactured according to current Good Manufacturing Practices (cGMP) and be commercially available, as for instance PROTASAN UP (ultrapure) chitosan salts from Pronova Biomedical.. The purity of chitosan will also influence the toxicological profile of the substance, and only the highest purity should be utilized in pharmaceutical applications.. The Ultrapure grade of chitosan salts, PROTASAN™ UP, has been tested for safety in various biological and physiological systems.

Keywords: chitosan, chitosan glutamate, PROTASAN, safety, toxicology, nasal tolerance, hypersensitivitiy, oral administration..

Introduction

Safety is one of the biggest issues for the commercialization of chitosan for veterinary or human medical applications. In the 1970's commercial production of chitosan for wound healing applications commenced. In 1997 the production of chitosan salts according to Good Manufacturing Practice (GMP) guidelines was established at Pronova Biomedical in Oslo. The development of new biomedical products involves several different aspects: properties of chitosan, production, quantity and quality, applications, and clinical effects [1]. However, the application of chitosan to human studies will involve regulatory approval in national and international authorities, such as the FDA in the US. Regulatory issues that are important in the commercialization of chitosan for biomedical uses are: Characterization and functionality, specifications of the product and analysis using validated methods. Stability of the compound is of prime importance. Reproducibility of the manufacture of the compound is very important which is ensured under a series of GMP guidelines. Documentation of not only the manufacture but also specifications and safety of the product is described in a Drug Master File (DMF), both in the US and Europe. Drug Master Files form the backbone of documentation that is required for registering a product, either as an active drugs or as an excipient. Finally, toxicology and safety covering basic studies and application-specific studies (route of

administration.) must be documented. This paper describes some relevant safety studies for chitosan salts produced as an ultrapure grade under the trademark PROTASAN™.

The basis for the production of chitosan salts (PROTASAN™) for biomedical and pharmaceutical applications is commercially available chitosan. Coming from a natural product, this starting chitosan can be contaminated with many impurities. The prime concern of regulatory authorities for products used in human medicine is the content of endotoxins and heavy metals. Microfiltration is used to remove insoluble compounds while ultrafiltration removes low molecular weight compounds. Currently chloride and glutamate salts of chitosan are being produced under the PROTASAN trademark.

Materials and methods

Chitosan glutamate and chitosan chloride are manufactured by Pronova Biopolymer as illustrated in the manufacturing scheme presented in Figure 1. The PROTASAN trademark covers several commercial grades of water-soluble chitosans for the pharmaceutical and biomedical industry. The materials are available as various salts of which chitosan chloride and chitosan glutamate are recommended. Each grade of PROTASAN is available as standard and ultrapure (UP) qualities.

Specifications for the chitosan glutamate or chitosan chloride used are shown in Table 1. The UP designation indicates an

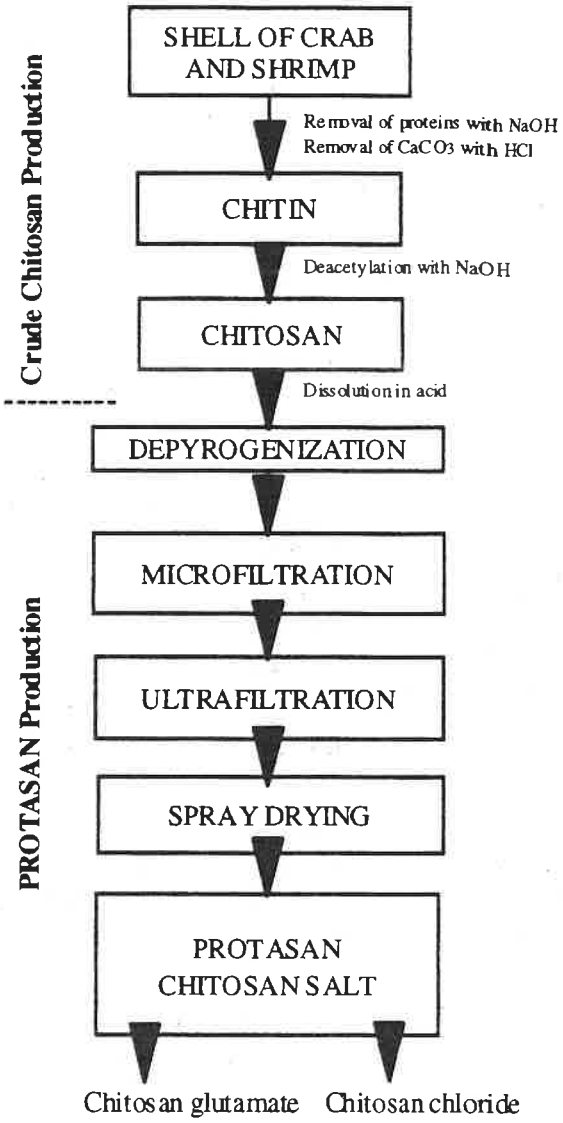

Figure 1: Manufacturing scheme for PROTASAN™ chitosan salts

Table 1: Specifications of PROTASAN™ used in the studies

Specification	UP G 110	UP G 210	UP Cl 110
Batch number	705-583-04	610-583-07B	310-490-01
Apparent viscosity	2.8 mPa.s	91 mPa.s	12 mPa.s
Intrinsic viscosity	0.7 dl/g	6.9 dl/g	ND
Degree of deacetylation	83%	83%	87%
Acid content	41% glutamic	40% glutamic	19% chloride
Loss on drying	4.9%	4.5%	4.6%
Insolubles	<0.1%	<0.1%	<0.1%
Heavy metals	<27 ppm	<26 ppm	<30 ppm
Endotoxin content	195 EU/g	625 EU/g	250 EU/g
Microbiology	< 1 cfu/g	<1 cfu/g	<1 cfu/g

ultrapure quality. The G stands for glutamate while Cl indicates a chloride salt. The 110 is a low viscosity chitosan while 210 indicates a medium viscosity . The chitosan salts were made up in physiological saline solution (0.9% NaCl), or in cell culture medium. Safety and toxicological studies were performed in accordance with applicable guidelines.

Results and discussion

Oral toxicity studies were performed in rats which received daily administration of chitosan glutamate solutions up to 600 mg/kg/day for 13 weeks. The results summarized in Table 2 indicate no toxicological effects were seen in any group. No real change in body weight was found between treated and control animals. Blood chemistry was also comparative between the 4 groups.

Table 2: Oral toxicity of PROTASAN™ UP G 210

Daily oral administration (gavage) to rats for 13 weeks

Group Number	Group Designation	Dose level (mg/kg/day)	Dose volume (ml/kg/day)	Dose conc. (mg/ml)	Results
1	Control	0	10	0	**No toxicological effects seen in any group**
2	Low dose	100	10	10	
3	Intermediate	300	10	30	
4	High dose	600	10	60	

An IV study was performed as a limit study investigating the effect of 25 mg/ml . Higher doses were toxic to animals. The cause of toxicity is most likely the aggregation of red blood cells by binding to chitosan resulting in blockage of capillaries. As seen below in Table 3, no toxicological effects were seen at this dose level.

Table 3: Intravenous toxicity of PROTASAN™ UP G 210 & UP G 110

One single intravenous administration to rats

Group Number	Group Designation	Dose level (mg/kg)	Dose conc. (mg/ml)	Results
1	Low viscosity (UP G 110)	25	5	**No toxicological effects seen in any group**
2	Medium viscosity (UP G 210)	25	5	

IP studies showed that a bolus injection of up to 500 mg/kg chitosan glutamate or chitosan chloride do not result in any toxicological effects evaluated 7 days after injection (Table 4). There was no change in body weight relative to control animals and no changes in physical or macroscopic appearance of the organs.

Table 4: Intraperitoneal toxicity of PROTASAN™ UP G 210 & UP Cl 110

One single intraperitoneal administration to rats

Group Number	Group Designation	Dose level (mg/kg)	Dose conc. (mg/ml)	Results
1	Low dose	100	5	**No toxicological effects seen in any group**
2	Intermediate dose	250	15	
3	High dose	500	25	

One of the prime applications of chitosan salts is in the field of drug delivery. The bioadhesive properties of chitosan can be used in nasal applications [2]. Safety of nasally administered chitosan glutamate was determined by treating rats with 0.5 or 1% chitosan glutamate solutions 3 times a day for 7 consecutive days (Table 5). Sections of the nasal cavities were stained with hematoxylin and eosin. Histological examination of the nasal mucosa indicated some increase in the thickness of the mucus layer in chitosan-treated animals (Figures 2 & 3). Goblet cells increased the production of mucus, not unlike the reaction occurring in other nasal irritation reactions. This reaction could also be due to the treatment technique itself. One of the most important findings, however, was that ciliated cells appeared normal, there was no de-ciliation of these cells.

Table 5: Nasal irritancy of PROTASAN™ UP G 210
Three daily intranasal administrations to the rat for one week

Group Number	Group Designation	Dose level (mg/rat/day)	Dose volume (µl/rat/day)	Dose conc. (mg/ml)	Results
1	Control	0	3 x 100µl	0	**Congestion in all groups. Increase mucus, not dose related. No toxic effect on ciliated cells**
2	Low dose	1.5	3 x 100µl	5	
3	High dose	3	3 x 100µl	10	

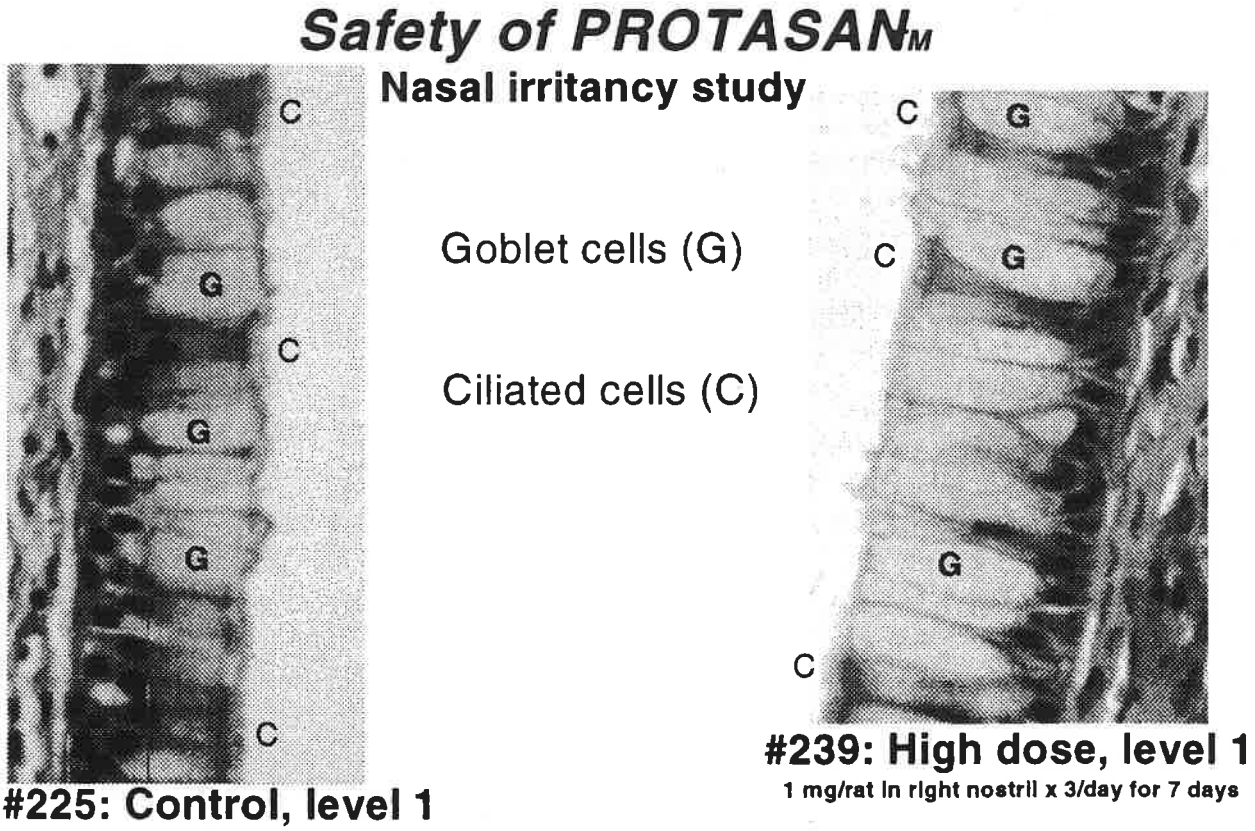

Figure 2: Effect of chitosan glutamate on rat nasal epithelia

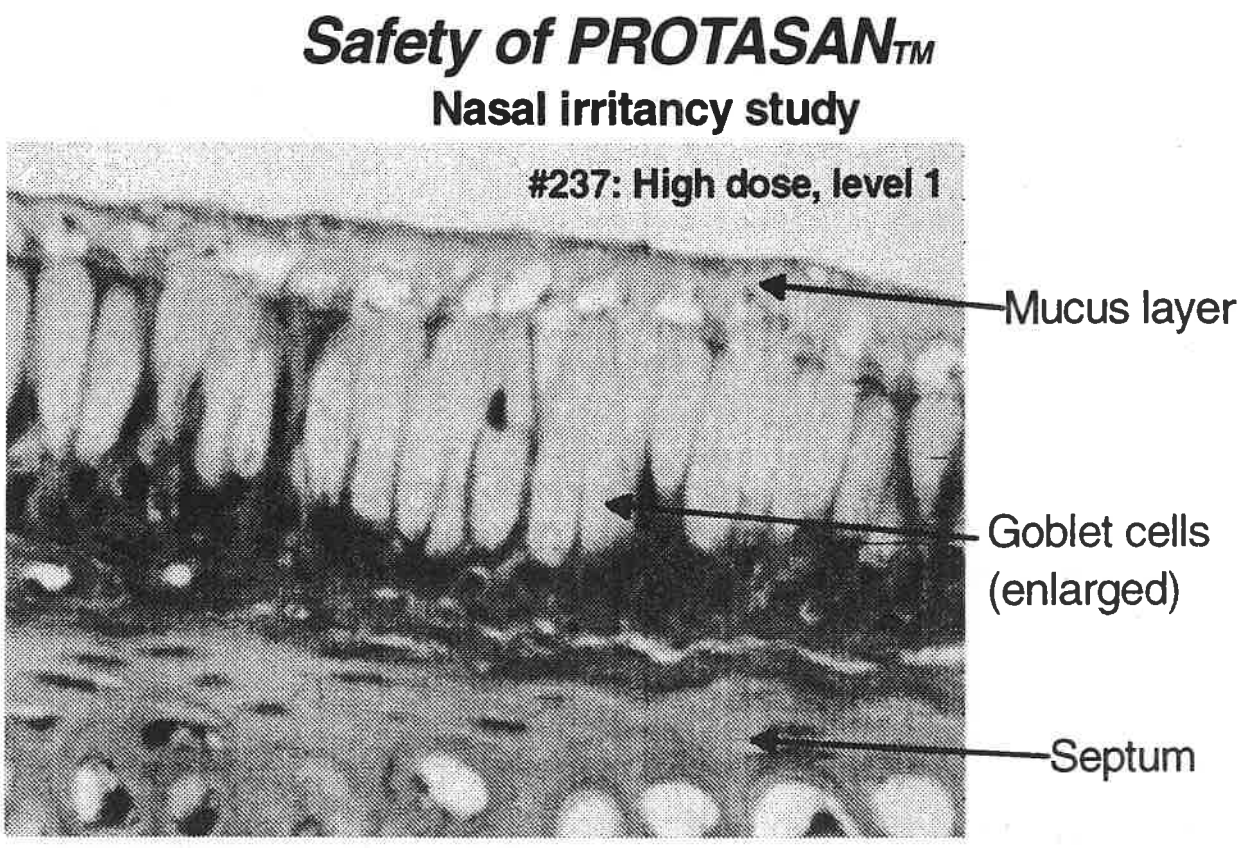

Figure 3: Effect of chitosan glutamate on rat nasal epithelia

Two hypersensitization studies using PROTASAN UP G 210 were performed. A Magnusson and Kligman test of the sensitizing potential in guinea pigs is a study in which the compound to be tested is intradermally injected to small areas on the back of the guinea pigs either with or without Freund's adjuvant. A topical application of a highly concentrated chitosan glutamate solution was given as the challenge at a separate site to the induction. Dichlorinitrobenzene was used as the positive control. The results in Table 6 show no hypersensitive reaction on application of the chitosan glutamate.

Anaphylactic shock is an antibody-medicated reaction leading to mortality. Guinea pigs were injected subcutaneously with chitosan glutamate as an induction. The challenge was given as an intravenous injection 21 days later. The positive control was ovalbumin which, on challenge, caused death in 8 of 10 animals. There was no mortality in the chitosan-treated groups and body weights were normal. There was some cyanosis directly after injection of chitosan salts, but this condition cleared by 4 hours after injection.

Table 6: Hypersensitization studies with PROTASAN™ UP G 210

Study	Induction	Challenge	Results
Magnusson & Kligman	1 mg/ml UP G 210 Intradermal 2 x 0.1ml -Freund's -0.9%NaCl 50:50 NaCl/Freund's	60 mg/ml UP G 210 Topical 0.5ml 48 hr.	**No mortality. body wt. not affected Irritation during induction No delayed hypersensitization (Positive control: 10/10 with DCNB)**

Anaphylactic Shock	10 mg/kg UP G 210 Subcutaneous Days 1 & 8	20 mg/kg IV Day 21	**No mortality in treated groups. Body wt. not affected. Cyanosis in 5/10, normal after 4 hr. (Positive control death in 8/10 using ovalbumin)**

The Ames test is an evaluation of the mutagenic potential of a compound. In this study chitosan glutamate was incorporated into the dishes used for culturing various strains of *Salmonella*. The study also evaluated the potential of an S9 mitocondrial extract to metabolize the chitosan into a mutagenic compound. The results in Table 7 show that chitosan glutamate induced no mutagenic effect up to a concentration of 5000 µg incorporated in each dish. The lack of mutagenicity was irrespective of the presence of an S9 mixture.

In vitro studies have shown that chitosan salts induce very little toxicity to cultured cells. In Table 7, the effect of a 24 hour incubation with chitosan salts on the mouse embryonal cell line 3T3 and the Chinese hamster lung cell line V79 were tested. Neither of these cell lines is considered cancerous or malignant.

Table 7: Other safety studies with PROTASAN™

Study	Dose	Schedule	Results
Ames test	Up to 5000 µg/plate UP G 210	±S9 activation TA98, TA100, TA1535, TA1537 & TA102 strains of *Salmonella*	**No significant increases in the number of revertants observed. 5000µg/plate toxic to TA98 & TA1537**
In vitro cell survival (Colony forming assay)	Up to 1 mg/ml	24 hr exposure 3T3 and V79 cells	**Little effect on cell survival.**

Conclusion

The evaluation of ultrapure chitosan salts has shown these compounds to be well tolerated in safety and toxicology studies. These data are important for a further evaluation of the usefulness and applicability of chitosan in biomedical and pharmaceutical applications. In addition, safety studies of the types presented here are necessary for regulatory approval of the use of chitosan salts in humans.

Oral applications of chitosan have been previously reported by others and the safety of orally administered chitosan has been reviewed by Weiner [3] and Hirano *et al.* [4]. We also show that long-term (13 week) administration of chitosan glutamate had no deliterious effect in rats. Further toxicological evaluation in animals and man have shown that chitosan glutamate has no deleterious effects on the nasal mucosa nor on mucociliary transport [5,6]. These findings are of importance for the development of chitosan in nasal drug delivery systems.

When chitosan was introduced as an industrial product in the early 1970's, the field of wound healing played a significant role in the initial commercial development of the biopolymer. For new pharmaceutical and biomedical applications of chitosan to be successful, studies like the ones presented here will be of importance. Moreover, regulatory issues, such as production process validation, quality control and product stability, will have to be addressed. In addition to characterization and functionality, the commercial manufacture of chitosan products for pharmaceutical use must also include product reproducibility and safety. PROTASAN salts are manufactured in accordance with GMP guidelines in order to insure quality control and documentation for commercial grades of water-soluble chitosan salts.

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Efficiency of High Molecular Weight Chitosan in Skin Care Applications

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Abstract
Chitosan and its derivatives have been known for many years as alternatives to synthetic polymers in hair care products, but only a few references describe the efficiency of these cationic polymers in cosmetic applications. Besides a well documented efficiency of a product, also the cosmetic acceptance, product safety and environmental aspects are corner stones in cosmetic concepts. Based on the definition of a cosmetic product, the care and protective effects on the skin are predominant targets of cosmetic development. Since emulsions are not simply mixtures but also contain a lot of ingredients various aspects of efficiency of High Molecular Weight (HMW) Chitosan (Hydagen® CMF) were investigated in four different in vivo and in vitro studies.
With the test design of the DAPPT (double application prolonged patch test) on volunteers, which represents an exaggerated form of the commonly used single application patch test, the improvement of skin compatibility was demonstrated. Supplementation of a basis cream with 0.1% active substance of Hydagen® CMF led to a reduction of skin reactions by approximately 70% in comparison to the placebo cream.
In a further in vivo study on volunteers a positive effect on the water resistance of sun protection emulsions could be shown by Hydagen® CMF which leads to a higher skin protection property of these products.
In vitro studies with the Bovine Udder Skin (BUS) model were performed to investigate the influence of Chitosan on the release of vitamin E (RRR-α-Tocopheryl Acetate) out of test emulsions into the horny layers of skin. Using the tape stripping method the results show that the release of the vitamin out of the verum emulsion with Hydagen® CMF was significantly increased.
The fourth study concerned the effect of Hydagen® CMF on the growth and adhesion of isolated skin cells. In contrast to other tested polymers, the incubation with Chitosan led to a stronger adhesion of fibroblasts (PF207 cell line) demonstrated by the MTT-assay after low and strong shear stress.
These positive effects demonstrate the efficacy of the biopolymer Chitosan as an ingredient of cosmetic emulsions and as raw material due to its outstanding biochemical characteristics.

Keywords
Chitosan, Hydagen® CMF, skin care, skin compatibility, UV protection, water resistance, skin penetration, adhesion, fibroblasts.

Materials and methods

Double application prolonged patch test (DAPPT)
Basis formula: An o/w-emulsion was tested without (placebo emulsion) and with 0.1% active substance (verum emulsion) of HYDAGEN® CMF.

The test design was developed by Jackwerth, 1997. Two plasters (Finn Chamber®) filled with the test emulsions (70µl on Scanpore®) were attached onto the backs of 18 healthy volunteers (6 male and 12 female, normal skin type) in accordance to the guidelines for human tests (Colipa Guideline, 1995). The 1st period of exposure was 48 hours. After this period the plasters were removed and skin reactions were controlled. The 2nd application was performed with identical substances on the respective application site for an additional exposure period of 72 hours. The reactions were clinically evaluated 6, 24, 48 and 72 hours after the final removal of the plasters (standardized lighting conditions, 2 day-light neon tubes: OSRAM L58W/12-Lumilux Delux). The parameters erythema, oedema, squamation and fissuration were assessed and documented (Frosch and Kligman 1979). From the individual reaction values obtained, the irritation sum values were calculated for each parameter, and for parameter combinations over all times of assessment and for all volunteers.

Determination of water proofness on volunteers

The study was performed with 6 volunteers with normal skin type and different light sensitivity. The products (emulsion with 0.1% HMW Chitosan and the corresponding placebo) were applied on two fields on the back (7x7 cm^2, 2mg/cm^2). After 15 min. incubation one site was showered representing 20 min. swimming. 20 hours after irradiation with UV-light, the MED (Minimal Erythemal Dosis) was determined for the two emulsions in comparison to unprotected skin.

Penetration study with the isolated perfused bovine udder:

The isolated perfused bovine udder model (Kietzmann et al. 1993, 1995;) was introduced in dermatopharmacology to study percutaneous absorption of xenobiotics. The experimental procedures were performed at SIMRED GmbH (D-30938 Großburgwedel). Details of the method are described elsewhere (Pittermann et al. 1995).

Adhesive tape stripping

After an exposure period of 3 hours (4g / 100 cm^2, once, non-occlusive) and dry cleansing the application site, ten succesive tape strips (Tesafilm® Beiersdorf AG, D-20245 Hamburg) of the stratum corneum were carried out. The stripped amount of stratum corneum was about 0.05 mg / cm^2.

Basis formula: o/w emulsions, with and without 0.1% active substance of HYDAGEN® CMF) were tested with vitamin E of natural sources (1% RRR-α-Tocopheryl Acetate), the content of water in the formulation is reduced by 1%.

Detection of vitamin E: The detection of vitamin E (Copherol® 1250) in the adhesive strips is based on the extraction of the analytes and the subsequent liquid chromatographic analysis of the obtained extracts.

The influence on fibroblast growth/adhesion:

Polymers: HYDAGEN® CMF, Polyquaternium 7 (P7), Polyquaternium 10 (P10) Soluble Collagen (SC), and Guar Hydroxypropyl Trimonium Chloride (GHTC).

Fibroblast cell line PF207: The cell line PF207 consists of fibroblasts, established from pig skin. The cells are grown in common culture dishes in DMEM/F12-Medium (3:1) with supplementation of specific growth factors.

MTT-assay: The assay is based on the cleavage of the tetrazolium salt MTT (Methyl-Thiazol-Tetrazolium) by viable cells. The reaction produces a water-insoluble formazan salt which must be solubilized with Dimethylsulfoxide for the quantitative analysis with an ELISA plate reader.

Test design: Cells are seeded in a definite density into culture vessels. On the 2nd day of cultivation the test substances are applied in different concentrations for a period of 24 hours. Afterwards the cells are washed under high pressure with phosphate buffered saline to induce strong shear stress before the incubation with the MTT dye follows.

Results

I. Improvement of skin compatibility in cosmetic formulations

The visible skin reactions consisted of erythema and squamations (Figure 1). The irritation score for the placebo cream reached 1.39 (erythema = 0.72; squamations = 0.67). In comparison to the variety of other formulas tested by the DAPPT up to now the value of 1.39 meets approximately the average of sum irritation scores. Supplementing the formula (equal 100%) with 0.1% active substance of HMW Chitosan reduces the irritation score for the parameter erythema and squamations significantly (28%).

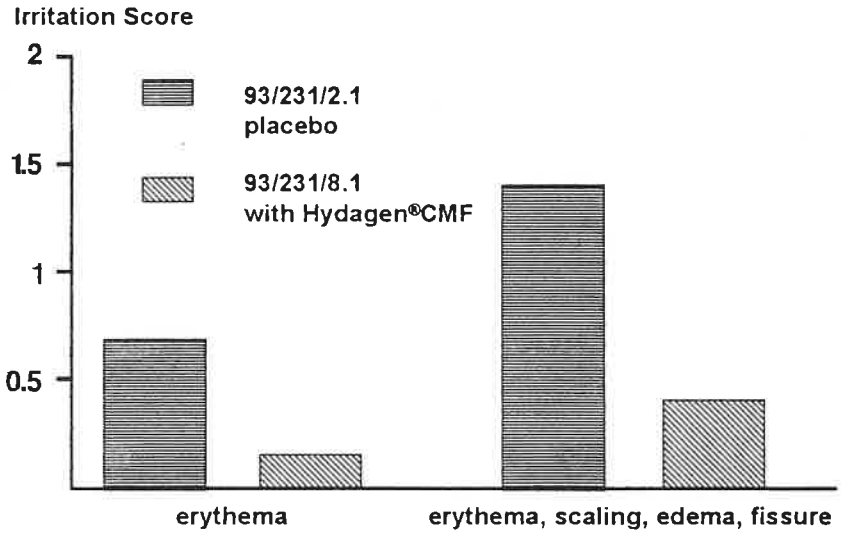

Figure 1: DAPPT: Significantly lower irritation scores with a cream formula containing 0.1% active substance HYDAGEN® CMF in comparison to the placebo cream

II. Enhancement of water resistance of formulations

Supplementation of the emulsion with Hydagen® CMF led to a higher SPF after washing in comparison to the placebo emulsion. The residual protection was 74.3% with Chitosan in contrast to 57.2% without Chitosan (s. Fig. 2)

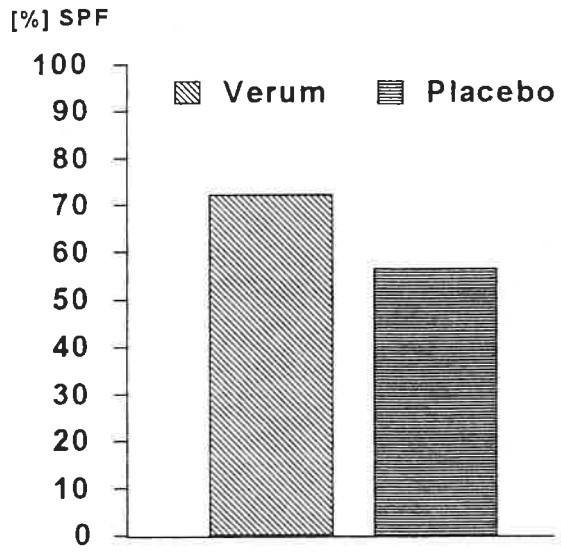

Figure 2: Increase of water resistance by a cream formula containing 0.1% active substance HYDAGEN® CMF in comparison to the placebo cream

III. Release of bioactive ingredients in formulations

The distribution of Copherol® 1250 obtained in the stratum corneum were different between both, the verum- and placebo emulsions (Figure 3). The average amount of Copherol® 1250 per strip released by the placebo emulsion was very low (mean: 0.45 µg / cm^2 stratum corneum = 21%) compared to the amount obtained by the verum emulsion (mean: 2.16 µg / cm^2 stratum corneum = 100%). The distribution of Copherol® 1250 after the verum treatment shows a steep gradient within the strips 1 - 3 compared to the slight gradient after the placebo treatment. Within the results of the strip 4 - 10 no gradient was observed after both treatments, however the amount per cm^2 stratum corneum released by the HMW Chitosan-containing emulsion remains on a very high level.

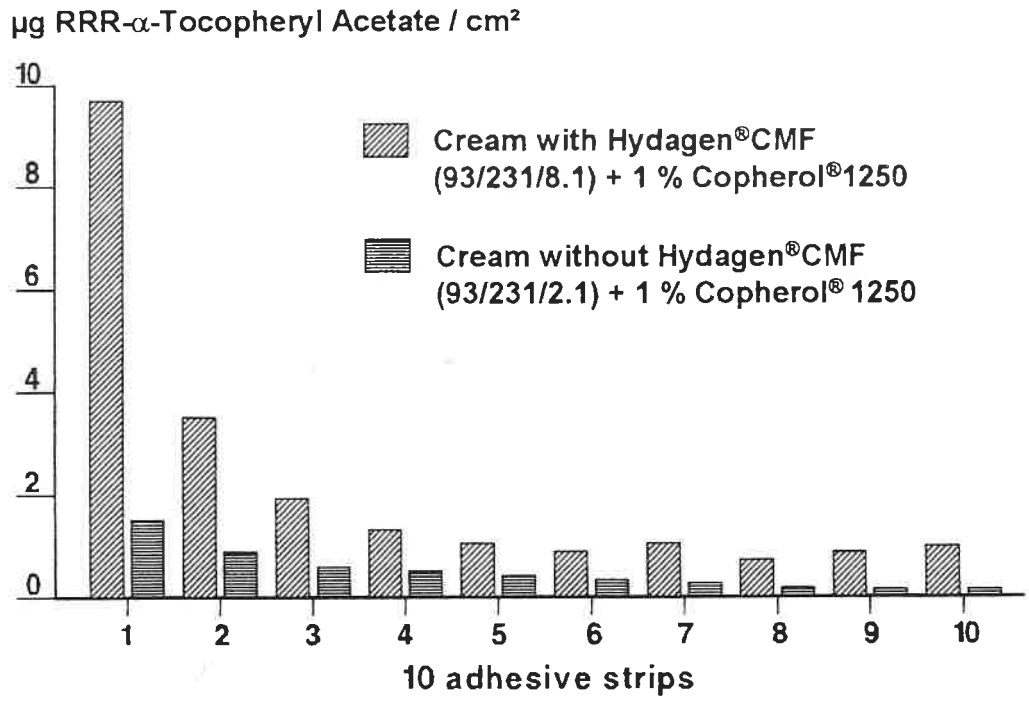

Figure 3: Release of RRR-α-Tocopheryl Acetate (Copherol® 1250) into the stratum corneum

IV. Effect on cell growth / adhesion

Test results are shown as dose-effect-curves which demonstrate the concentration dependent effect of the polymers on the cell growth and the adhesion respectively. Performing a test design with low shear stress to PF207 cells no positive effect on cell growth / adhesion after treatment with the polymers P 7, SC, P 10 and GHTC (0.001% and 0.01% active substance) was found. On top of this, toxic effects with 0.01% GHTC and P 10 became obvious. The polymer P 7 showed negative effects in all concentrations. In contrast to these effects HMW Chitosan (0.01% active substance) induced a slight increase of vital, adherent cells (results not shown). The results of the study with strong shear stress show that the number of adherent cells in the control cultures not-treated with polymers was decreased by approximately 50% (results not shown). In relation to the untreated control values the treatment with 0.01% active substance of HYDAGEN® CMF led to a 35% higher cell number (Figure 4). In contrary the treatment with the other polymers caused no positive effect on cell adhesion.

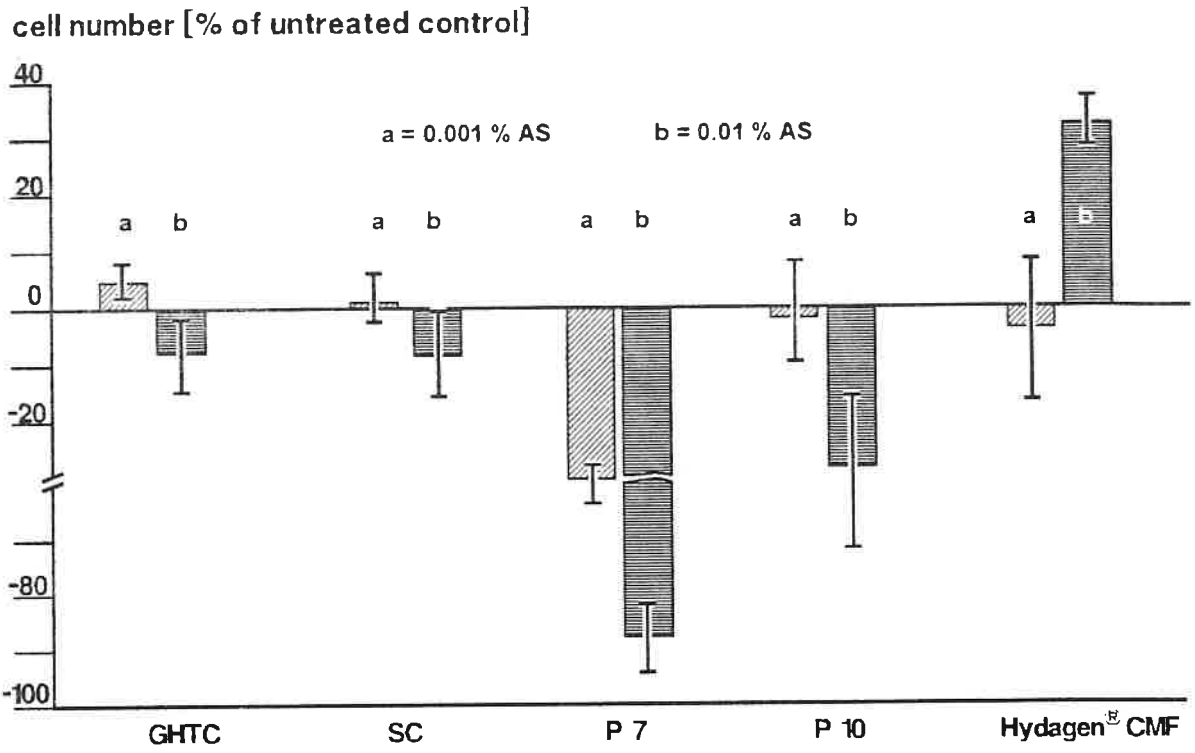

Figure 4: Influence of different polymers (Guar Hydroxypropyl Trimonium Chloride (GHTC), Soluble Collagen (SC), Polyquaternium 7 (P7), Polyquaternium 10 (P10), HYDAGEN® CMF) on the growth/adhesion of the fibroblast cell line PF207. Quantification of adherent, vital cells exposed to strong shear stress

Discussion

The efficacy of the cationic biopolymer HYDAGEN® CMF was investigated either as an ingredient in cosmetic formulations or as raw material. A cosmetic cream supplemented with 0.1% active substance of HYDAGEN® CMF was used in the in-vivo test DAPPT on volunteers to study the effect on skin compatibility in comparison to a placebo cream by exaggerated exposure conditions.

The test is designed to differentiate mild leave-on products which did not differ in the conventional patch test. The supplementation reduces the irritation score for the parameter erythema and squamations by approximately 70%. The biological basis for this effect is not known but one may assume a positive influence on the formulation components in addition to the known film-forming capacity of HYDAGEN® CMF (Wachter and Stenberg 1996). The result indicates the use of Chitosan as an ingredient especially in the field of products for impaired or sensitive skin.

Furthermore, HYDAGEN® CMF increases the water resistance of sun protection emulsions. In this case the excellent film-forming ability leads to a higher protection against UV irradiation.

The expectations on a film-forming product in cosmetics includes also other positive effects e.g. the availability of bioactive ingredients such as vitamins. The possible penetration enhancement of vitamin E by HYDAGEN® CMF was assessed in an in-vitro study using a perfused skin model. After a use-relevant application and an exposure period of 3 hours the release of RRR-α-Tocopheryl Acetate was measured by means of the adhesive tape stripping method. It is obvious that the HMW Chitosan-containing emulsion increases the release of cosmetic ingredients such as RRR-α-Tocopheryl Acetate. Under the influence of HMW Chitosan, the vitamin penetrates the horny layers easely e.g. a significant greater amount (approximately 80%) was found in the stratum disjunctum and stratum compactum compared with the placebo treatment. The reservoir capacity of the stratum corneum is filled up more rapidly if the formula contains HYDAGEN® CMF. According to Schaefer and Redelmeier (1996) the increased release of ingredients act at the level of stratum corneum. The molecular basis can be attributed to: (i) an increase in the partitioning of ingredients into the stratum corneum; (ii) an increase in the diffusivity of the ingredients into the stratum corneum, and (iii) a change in the penetration pathway. Since the penetration pattern of HYDAGEN® CMF into the stratum corneum is unknown, one can not distinguish between these possibilities. However, concerning the alteration of the barrier function after topical application, one may assume positive synergy occurs between the lipophilic vitamins and Chitosan.

A wide variety of Chitin based products for wound dressing like fleeces, gauzes, and powders exist today. Key factors of efficacy of chitosan in this relation are the incorporation into the extracellular matrix and the activation of different cell types (Muzzarelli 1996, Gorbach et al. 1994). Especially for fibroblasts, an activation of collagen deposition and positive effects on cell adhesion are demonstrated (Damour et al. 1994, Shahabeddin et al. 1990). Furthermore the film-forming capacity enables wound coating and in consequence a protection against microbiological contamination. The relevant in-vitro studies prove the positive effect of HYDAGEN® CMF as raw material on the adhesion of the fibroblast cell line PF207 in contrast to the effects of other tested polymers.

This is a specific property of HMW Chitosan and not a general characteristic of polymers. The molecular background might be an enhanced secretion of collagen or an improvement of adhesion by specific structures which serve as adhesion mediators. The molecular structure of Chitosan differs concerning its repeated units in comparison to other polymers. Glycosamin units are part of natural biomolecules in the human dermal layers as there are glycosaminoglycans and glycoproteins in the extracellular matrix of the fibrous tissue.

Conclusion

Four different in vivo and in vitro studies were performed examining the properties of HYDAGEN® CMF in skin care applications. Summarizing the results the cationic biopolymer offers various positive effects as a valuable ingredient in skin care products. Besides improvement of skin compatibility of emulsions and increasing skin protection against UV-irradiation by a higher water resistance, it positively influences the bioavailability of lipophilic ingredients such as vitamin E, and it enhances the adhesion of fibroblasts in culture. These outstanding properties are based on the specific biochemical characteristics of HYDAGEN® CMF.

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WOUND HEALING DRESSINGS MODIFIED BY CHITOSAN

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Abstract

A search for the new effective wound healing dressings for special applications is concentrated more and more on the naturally originated materials.The combination of homogenated human placenta with selected forms of chitosan was a base for research.

The properties and behaviour of original wound dressing modified by microcrystalline chitosan are discussed.

Keywords: wound healing dressings, human placenta, microcrystalline chitosan, improvment of surgical susceptibility

Introduction

A search for the new effective wound healing dressings for special applications is presently more and more connected with naturally originated materials.The modern wound healing dressings should meet several requirments as follows:

- universality
- sterility or/and antibacterial & bacteriostatic action
- elasticity
- hydrophilicity
- none alergic
- suitable structure
- simplicity in application.

The presently exisiting wound healing dressings are as follows:

- conventional as bandages

- biological as own or animal skin,collagen forms
- synthetic as foams, sponges or film made from synthetic polymers such as polyurethanes
- mixed.

The collagen containing wound dressing under a name of Choriospon ® produced on the base of homogenated human placenta by the Blood Bank, Katowice, Poland (1) is characterized by several advantages such as biostacity, acceleration of cell granulation process, reduction of pain and usefulness for long healing or non - healing wounds, complicated surgical wounds or for transplants. However, an insufficient surgical susceptibility of above wound dressing concerned with non - elasticity as well as low mechanical tenacity was affected for its narrow application area. The application of chitosan forms such as a microcrystalline chitosan for modification of homogenized human placenta was resulted in elaboration of modern wound healing dressings (2 - 3).

The aim of this research was to present some aspects of the modification of homogenized human placenta, including the properties of wound healind dressing.

Materials and methods

A human placenta sterilized by γ-radiation system or after liophilization produced by the Blood Bank, Katowice, Poland was used in this research.Microcrystalline chitosan (MCCh) prepared according to original methods (4) at the Institute of Chemical Fibres, Lodz, Poland in a form of gel-like dispersion with properties presented in Table 1 was used for modification of wound dressing.

Table 1.Some properties of microcrystalline chitosan used

Symbol of sample	Polymer content , wt%	Mv , kD	WRV ,%	DD ,%
M 1/2	3.52	345.0	990	85.6
P 6/6	3.54	27.0	920	85.6

The wound healing dressings basing on a homogenized human placenta modified by microcrystalline chitosan were produced according to the original methods (2 - 3). All analytical methods used in these studies were typical for chitosan and wound healing dressings.

Results and discussion

A modification of homogenized human placenta by microcrystalline chitosan (2 - 3) was solved all unbeneficial behaviour of wound healing dressing of Choriospon ^{R} with some possitive effects on its biological action.Microcystalline chitosan gel-like dispersion used for modification was affected at first of all on the sorption behaviour of produced wound dressings (Table 2).

Table 2. Water retention values (WRV) for different wound healing sponges

Type of dressing	WRV, %
Choriospon ^{R}	320
Choriospon M ^{x}	580
MCCh	380

x - modified by MCCh of M1/2

The application of microcrystalline chitosan as the modifying component to a wound dressing was changed its porous structure to obtain a higher sorption ability (Tab. 2). At the same time a modified wound healing dressing is characterized by much better mechanical parameters, including tenacity,elongation and elasticity. A comparison of some properties of homogenized human placenta dressings are shown in Table 3.

Table 3. Some properties of tested wound healing dressing

Type of dressing	Minimal thickness of dressing , mm	Minimal specific density dressing ,g/sq.m	Mechanical properties in		Elasticity	Suscepti-bility for rolling up
			dry	wet		
Choriospon ^{R}	6 -7	500	+	-	-	-
Choriospon M	2-3	100-250	++	++	++	++

A modified wound healind dressing containing selected forms of microcrystalline chitosan is characterized by very good mechanical properties, especially elasticity in dry and wet state as well as ability to be rolled up.This dresssing absorps also for a short time of several seconds all liquid from a covered surface.

A preliminary medical test shown a surgical susceptibility of discussed modified wound dressings.The medical tests for wound healing are presently carried out.

Conclusions

1. A new wound healing dressings basing on the homogenated human placenta and microcrystalline chitosan were prepared by optimization of used composition and preparing process parameters.
2. Modification of human placenta basing wound healing dressings by microcrystalline chitosan has caused several benefits including improvment of its mechanical properties, elasticity and sorption behaviour.
3. A preliminary medical tests shown a complete surgical susceptibility of microcrystalline modified wound dressings.

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Study of carboxymethyl-chitin and hydroxyapatite composite for bone repairing

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(Abstract)

Chitin has been applied for some medical materials such as hemostatic and wound healing materials, and its fine results were reported clinically.[1)]Fundamental studies of the bio-degradable suture thread and the carrier of drug delivery system as the application of chitin and its derivatives have been studied.

Tokura et al studied physiological activity of CM-chitin(CM-ch), which is water-soluble chitin derivative including its macrophage activation, affinity for myelocytes and metabolic phenomena.[2)] The biological safety in terms of acute toxicity and mutagenicity is also confirmed. [3)]

Studies of bone tissue repairing have been done in the field of orthopaedics and oral surgery long since. Recently, bio-degradable materials, which are made from some synthetic polymers and the collagen derived from animals, have been applied to clinical treatments.[4)]

But difficulties in handling, the limitation in applicable cases, and the unstable efficiency of the treatments are indicated.[5)]

On the other hand, hydroxyapatite(HA) is a calcium phosphate compound and is known to chemically bond to bone tissues [6)].

In order to solve these problems existing in bone repairing materials, we consider that it is necessary to study more functional bone repairing material. By using CM-ch which is recognized to be biologically safe, we prepared CM-ch · HAg composites by the unique method and examined the effects in bone repairing by animal experiment.

Keywords: carboxymethyl-chitin, hydroxyapatite, bone repairing, composite material, animal experiment

(Materials and methods)

·Preparation of CM-ch·HAg composite

CM-ch(DS:0.7, M.W:300,000, DAC:30%) material was dissolved in distilled water at room temperature. After insoluble residues were eliminated by a centrifugation, the freeze-dried powder from the solution was prepared. Having prepared a 3wt% aqueous solution, 20wt% HAg was mixed and stirred. The mixed solution was poured into vessels and frozen quickly in dry ice bath, and then freeze-dried. The CM-ch·HAg composite was prepared by heating treatment in a vacuum(160℃).

·Microstructure of CM-ch·HA composite

The internal structure is 3-dimensional porous mesh-like and the pore size is controlled to be smaller than 100μm. HAg(*60~150μm in diameter) exists at intervals of 200 ~ 300 μ m in the composite(Fig.1).

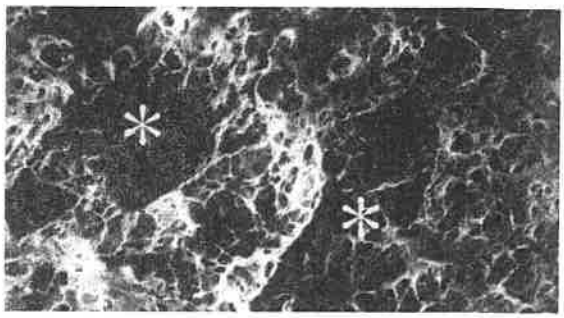

Fig.1 Scanning electron micrograph
Scale bar=100μm

·Surgical Procedures

New Zealand White rabbits(12weeks,3kg) were used. After they were anesthetized by pentobarbital sodium solution(0.5ml/kg), Approximately a 3cm incision was made over the proximal epithelium of the femur and the medial side of bone was exposed. Three holes(4mm in diameter, 3mm in length) were made with a drill at 5 mm interval. Then the following materials a)CM-ch·HAg, b)CM-ch-sponge, and c)HAg sterilized beforehand by γ radiation, were inserted. The wounds were closed with a conventional suturing.

·Preparation of undecalcified sections

The rabbits were sacrificed 2, 4, and 8 weeks after implantation and tibiaes including the materials were harvested.

Each tibiae was cut into small parts and dehydrated in alcohol, and then such specimens were embedded in the resin . Each block was sectioned by micro-cutting unit (BS3000N,EXAKT) at a thickness of 20 μm perpendicular to the long axis of the tibiae(Fig.2). Finally, the ground sections were stained with toluidine-blue solution

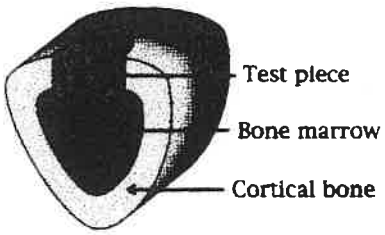

Fig.2 Schematic diagram of sectioned surface

·Histological examination

The degree of bone repairing at the implanted sites was quantified according to the following two methods.

(1)Extension ratio of repaired bone(%)

The lengths(L1,L2) of repaired bone which expanded from both hosts, were measured at the central position and summed up. Then the percentage of lengths(L1+L2) to L3 was calculated by the following formula.

Extension ratio of repaired bone(%)=[(L1+L2)/L3]×100

(2)Reparation ratio of bone thickness(%)

The thickness(T1) of repaired bone was measured at the central position.

Then the percentage of thickness(T1) to the average thickness(T2) of both hosts was calculated by the following formula.

Reparation ratio of bone thickness(%)=(T1/T2)×100

(Results and discussion)

The CM-ch · HAg composite showed high blood absorbing property and was tightly fixed in the bone defect without flowing out during periosteum suturing, while HAg were easy to flow out of the bone defect by bleeding from the bone marrow cavity.

·Quantitative analysis of bone repairing

(1)Extension ratio of repaired bone(%)

Table.1

	Implantation period(week)		
	2	4	8
a)CM-ch·HAg composite	72.2	100	100
b)CM-ch-sponge	44.9	86.7	98.5
c)HAg	69.3	78.7	91.7
d)Blank	63.2	97.8	100

Data are means(n=3)

(2)Reparation ratio of bone thickness(%)

Table.2

	Implantation period(week)	
	4	8
a)CM-ch·HAg composite	105.0	101.4
b)CM-ch-sponge	51.4	43.3
c)HAg	73.5	66.1
d)Blank	58.1	44.4

Data are means(n=3)

·Light microscopic observation

a)CM-ch·HAg composite

Although a slight inflammatory cells infiltration was observed at 2 weeks after implantation, most inflammatory cells disappeared in 4 weeks after implantation and new bone formation was observed surrounding HAg(50~300μm interval). Slight condensation of HAg was seen partially, but in most area new bone formation including HAg was observed(Fig.4a). Furthermore, the data of extention(Table. 1) and thickness(Table.2) were more favorable than other materials, and the outflow of HAg could be scarcely seen.

b)CM-ch-sponge

CM-ch still remained in the bone defect at 2 weeks after implantation and intensive inflammatory cells infiltration was observed. For this reason, the extension of repaired bone was shorter than other materials(Table.1). But after 4 weeks, remaining CM-ch and inflammatory cells disappeared, and the thickness of repaired bone was similar to that of Blank cases(Fig.4b).

c)HAg

Although active new bone formation was observed around the HAg neighboring the host bone at 2 weeks after implantation, HAg gradually flowed out and tended to be condensed in the center of bone defect(Fig.4c).

d)Blank

Newly formed bone was observed extending toward the center of bone defect at 2 weeks after implantation, and both sides of host bone were connected in the deeper area of the bone defect at 4 weeks later. But bone formation in the upper area of bone defect was inhibited due to the presence of fibrous connective tissues(Fig.4d).

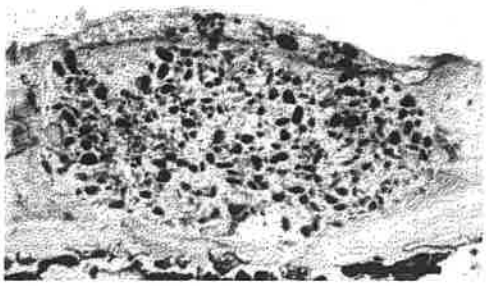

Fig.4a

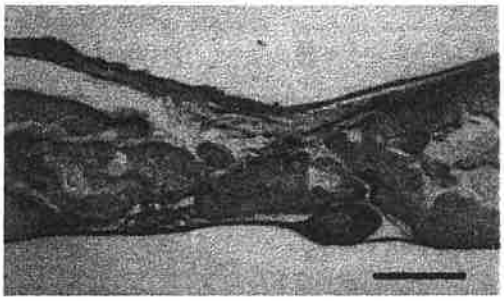

Fig.4b

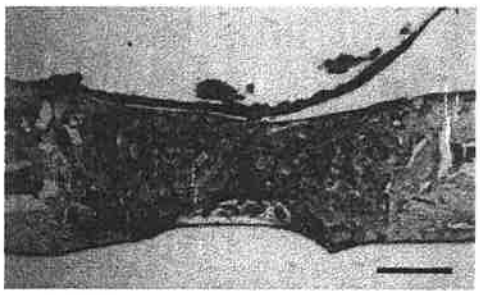

Fig.4c

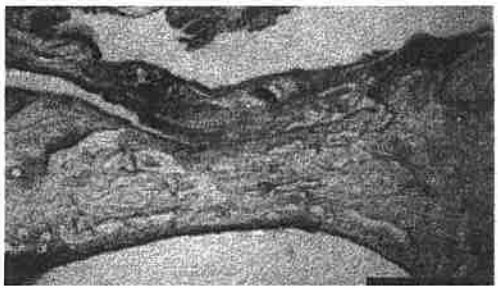

Fig.4d

Histologic appearance at 8weeks afer implantation
Scale bar =1.0mm

(Conclusion)

Bone defects which are large or contacting with soft tissues are difficult to repair with HAg contrary to surgeons'expectations.

It would be necessary to lead adequate bone repairing that osteoconductive materials like HAg must be firmly fixed while keeping suitable intervals by the carrier which can store osteoblast-like cells.

Muzzarelli et al reported that a chitosan derivative had an osteoconductive property.[7] In this study, we could not observe the osteoconduction of the CM-ch sponge material, but found the CM-ch to be untoxic and to have digestible properties in bone tissues. The results of this study suggest that porous CM-ch·HAg composite is a functional material which could act as a scaffolding of osteoblast-like cells, a barrier to ingrowth of fibrous connective tissues.

We are planning studies to develop the CM-ch·HAg composite material which can be used in large bone defects and in the gap between host bones and metallic or ceramic implant devices.

(Acknowledgment)

We thank KOYO CHEMICAL CO.,LTD.(Tokyo,Japan) for supplying CM-chitin raw materials.

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SOME ASPECTS OF MICROCRYSTALLINE CHITOSAN HYDROGELS OINTMENT BASE FORMULATION

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Abstract

The effect of the microcrystalline chitosan in the form of gelatinous water dispersion and film on the release of diclofenac salts (DS) has been studied in vitro. The methylcellulose has been applied as a model gel creative substance. The quantity of the released substance was determined by ultraviolet spectroscopy and by measurement of electric conductivity. The rate of drug release from the hydrogels-systems through membrane is lower than that for the film-systems immersed directly in a water bath. It has been found that this process can be described at a first order rate. The slow controlled release action of DS in a case of microcrystalline chitosan hydrogel was observed.

The release rate of zinc ions from microcrystalline chelates was decisively much slower in comparison to dissolving rate of individual metal salts. The release of Zn^{2+} from the microcrystalline chitosan chelates can play an important role especially for medical use. It was found that microcrystalline chitosan hydrogel may be used as a base substance for active substances of local applied.

Keywords: Microcrystalline chitosan hydrogel; Diclofenac salts; Zinc ions; Controlled release

Materials and methods

Microcrystalline chitosan (MCCh) in the form of gelatinous water dispersion with a polymer content of 3.0 % wt., water retention value (WRV) of 1030 %, average molecular weight of $\bar{M}w = 2.02 \times 10^5$, and deacetylation value of 85.7 % prepared according to the original method[1] and methylcellulose (MC) with a viscosity of 110 cP were obtained from Institute of Chemical Fibres, Łódź, Poland. Methylcellulose gel (3 %) has been obtained *ex tempore* by mixing of the powder with water. Diclofenac sodium (DNa) and triethanolamine (TEA) were purchased from Sigma Chemical Co. Diclofenac acid (DA) was obtained from Pharmaceutics Laboratory „BIOCOM" Rzeszów, Poland. Diclofenac IV-order ammonium salt (DTEA) was obtained by manually mixed diclofenac acid with amine. The diclofenac acid - to - amine concentration ratio was 1 : 1. Tomofan - regenerated cellulose, type PFT, 35 g/m^2, was obtained from „Wistom", Tomaszów Maz., Poland. The other used chemicals were: 1,2-propylene glycol and Zinc nitrate Hexahydrate (Fluka Chemie AG), glycerol (Polish Chemical Reagents, Gliwice, Poland), commercial diclofenac gel - Olfen-gel (Mepha Ltd, Switzerland).

The incorporated drugs to MCCh and MC gels were diclofenac salts (DS) at 1 % concentration and Zn (II) nitrate salt (only to MCCh) at 10 % concentration. The kinetic of water released from gelatinous dispersion (MCCh) and hydrogel (MC) before and after charged drugs has been investigated. Weighed amounts (ca 1.0 g hydrogel) dried at 37 °C were weighed at appropriate intervals time (weight with accuracy of 0.001 g).

The effect of microcrystalline chitosan both in the form of gelatinous aqueous dispersion and film on the release of DS and Zn (II) has been studied. Microcrystalline chitosan hydrogel - 2 g, charged drug - 0.02 g DS, 0.2 g Zn (II) was spread on a Teflon plate and the water was evaporated at room temperature. After evaporation of the water, xerogel film was obtained. The piece of xerogel film with dimension of 4 cm × 4 cm was immersed directly in distilled water (1000 ml) contained in a double-walled glass beaker thermostatted at 37 ± 0.1°C.

The liberation of diclofenac salts from microcrystalline chitosan aqueous dispersion, methylcellulose hydrogel and comparative commercial diclofenac gel (Olfen-gel) was determined by membrane method to water. The media were stirred at 60 rpm. The samples were periodically removed and analysed spectro-photometrically at a maximum absorbance (DS $\bar{\nu} \approx 36\times10^3$ cm^{-1}; Zn (II) $\bar{\nu} \approx 33\times10^3$ cm^{-1}, Specord M-40 Spectrophotometer, Zeiss). The received sample was turned back at once into beaker after measurement concentration of active substance. The results of measurements were calculated as dependence concentration of substance in water on the release time.

The quantity of the diffused substance was also determined by measurements of electric conductivity (Microcomputer Conductivity-meter CC-317, Elmetron, platinum electrode EPS-2 ZE, Eurosensor, Gliwice, Poland). Corrections were made for electric conductivity water.

The influence of hydrophilizing agents on release rate of drug from modified microcrystalline chitosan hydrogel (MCCh mod.) was studied. 1.2- propylene glycol (PG) and glycerol (G) have been mixed with microcrystalline chitosan hydrogel in proportion: 1 : 1 : 8.

Results and discussion

The idea of the application of chitosan as auxiliary substance in direct tableting[2, 3] has been extended upon its usage, after solving in lactic acid, as a base for semisolid dosage forms[4]. Microcrystalline chitosan (MCCh) existing as a new microcrystalline form of standard chitosan is a special functional polymeric material[5] because contains free of amine groups. The new form retains its microcrystalline character above pH > 6.5. MCCh in the -NH_2 form has highest biological effectivity and may be used as a vehicle in trials of preparing the dermatological gels in consistence of ointment. Several possible MCCh medical and pharmaceutical applications have already been studied [5, 6]. Based on the unique behaviour of microcrystalline chitosan, especially in a dispersion form, new application concerned pharmacy and medicine is presented.

Kinetics release of water

The process release of water from hydrogels can be correct presented by equation:

$$m_t = m_\infty (1 - k \cdot t^a) \qquad (1)$$

where: m_t - quantity released of water in time t,

m_∞ - total quantity of water in preparation,

k i a - constants of equation.

The great values of coefficient of correlation (Tab. 1) confirm this equation. The calculated k values on a basis this equation prove of slower release of water from MCCh-hydrogel than from MC. The release rate of water from MC was compared with the evaporation rate of water. Only small differences between the release rate of water

from hydrogels charged DS and hydrogels without any drug were observed. The release of volatile substances from commercial gel (Olfen) was considerable faster.

Table 1. Coefficient of correlation and release parameters of water from hydrogels

No.	Species of gels	k, min^{-1}	$\pm\Delta k, min^{-1}$	a	$\pm\Delta a$	r^2
1	MC	0.020	0.004	0.80	0.04	0.9976
2	MCCh	0.014	0.008	0.94	0.21	0.9944
3	Olfen gel	0.048	0.006	0.64	0.02	0.9923
4	MCCh + DNa	0.013	0.006	0.83	0.06	0.9967
5	MC + DNa	0.019	0.004	0.76	0.04	0.9976
	H_2O	0.022	0.003	0.78	0.11	0.9980

Kinetics release of diclofenac derivatives

The dependence of drug concentration in water bath on the time can be presented at a first order rate. In order to understand the mode of release of drug from the film as well as from hydrogels, the release data were fitted to the following equation:

$$C_t = C_\infty - \left(C_1 \cdot e^{-k_1 \cdot t} + C_2 \cdot e^{-k_2 t}\right) \qquad (2)$$

where: C_1, k_1, C_2, k_2 constants of equation.

The changes of electric conductivity in the time of release DS from MCCh-film to water are given in Fig 1. Two partial functions describing the changes concentration of DNa have been presented. The function 1 presents quick and great changes of concentration of DNa (release DNa from surface of film). The function 2 presents small changes of concentration of DNa (diffusion DNa from interior of film). The function 3 presents theoretical dependence of electric conductivity describing release DNa from MCCh-film to water.

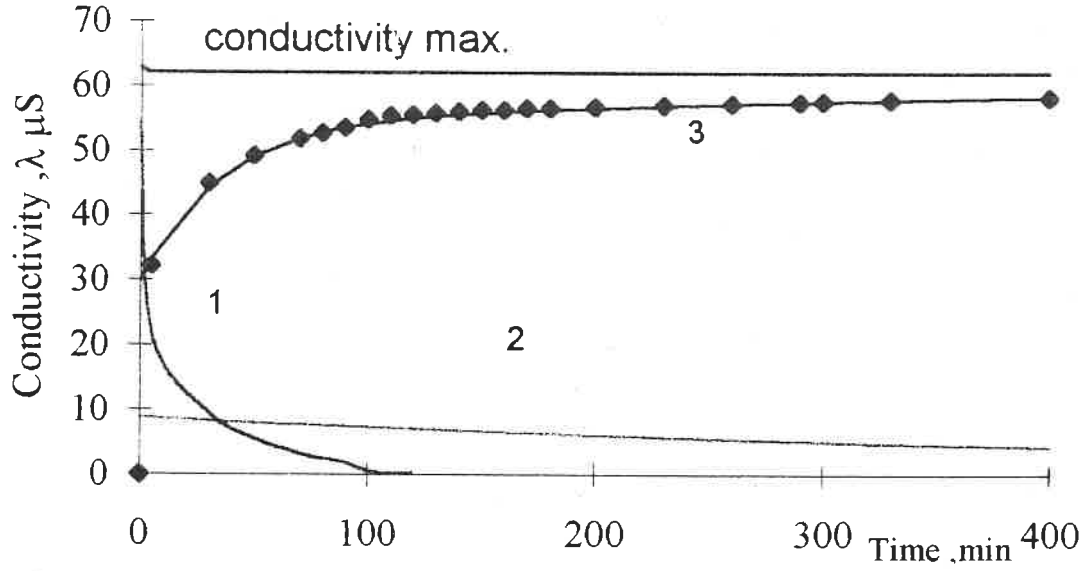

Fig. 1. The changes of electric conductivity solution against time release DNa from MCCh-film

The different release rate of drug has been obtained in dependence on method applied. The release from film directly immersed in water follow faster than from hydrogel through membrane. In contrast with the rapid release of DTEA from MCCh-film, sustained release was obtained from the MCCh-hydrogel (Fig.2).

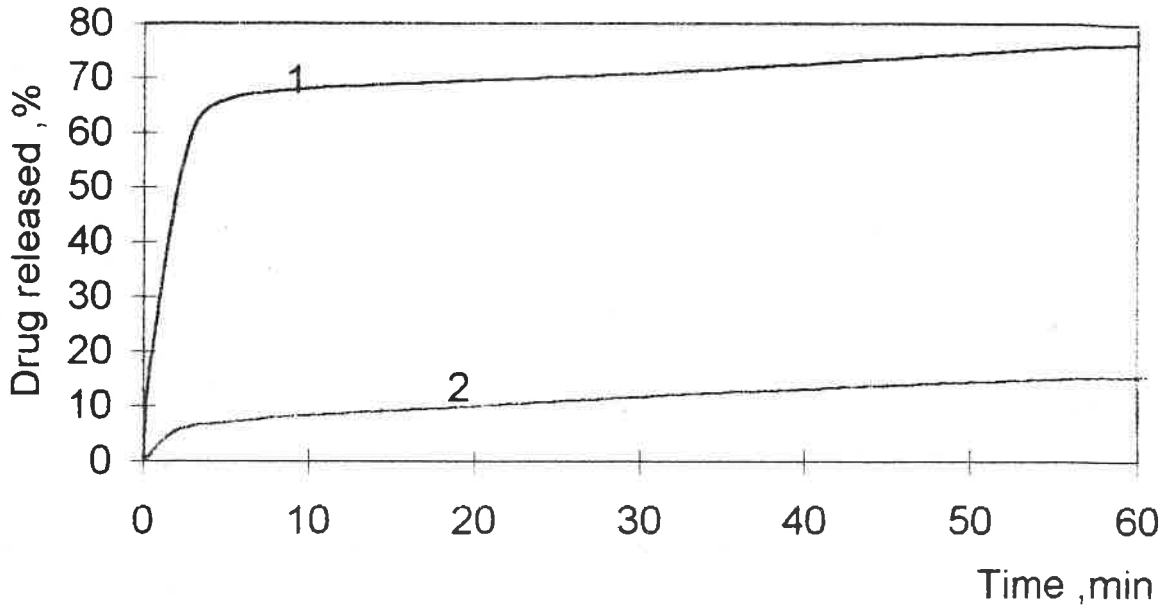

Fig. 2. The influence of investigation method on release rate DTEA: 1- from MCCh-film; 2 - from MCCh-hydrogel

The release parameters of drugs (DS) from MCCh-hydrogel and film are presented in table 2 and 3.

On the basis of results presented in Tab.2 can be confirmed that majority of DS (nearly 90 % of DNa and above 60 % of DTEA) was released in a first stage (difficult to measurement). In the studied stage the different release rates of drugs have been obtained. Release rate of DNa from MCCh-film depend upon the presence of hydrophilizing agents. 1.2-Propylene glycol and glycerol additives inhibit release of DNa in the second stage. The release of DS from MCCh-film without any hydrophilizing agents is faster in comparison with modified MCCh - film (MCCh mod).

Table 2. Calculated results of release rate of diclofenac derivatives from MCCh-film

No	Species of dosage form	C_1 $\pm\Delta C_1$	$k_1 \times 10^3$ $\pm\Delta k_1 \times 10^3$	τ	r^2
		,%	,min^{-1}	, min	
1.	MCCh + DNa	9.6 0.8	8.0 0.4	125	0.9974
2.	MCCh mod. + DNa	9.2 0.03	0.96 0.04	1040	0.9990
3.	MCCh + DTEA	34.4 1.3	6.2 1.0	161	0.9930

Table 3. Calculated results of release rate of diclofenac derivatives from MCCh-hydrogels

No.	Species of dosage form	C_1 $\pm\Delta C_1$,%	$k_1\times10^3$ $\pm\Delta k_1\times10^3$,min^{-1}	τ , min	C_2 $\pm\Delta C_2$,%	$k_2\times10^3$ $\pm\Delta k_2\times10^3$,min^{-1}	τ ,h
1.	MCCh + DNa	25.8 0.8	25.5 1.2	39	73.9 1.1	0.53 0.06	31.4
2.	MCCh mod. + DNa	-	-	-	96.9 1.2	0.76 0.03	21.9
3.	MCCh + DTEA	15.9 0.6	11.7 0.8	85	77.9 6.2	0.28 0.07	59.5
4.	Olfen gel	-	-	-	98.9 0.8	0.30 0.01	55.6
5.	MC + DNa	-	-	-	96.8 1.0	0.55 0.02	30.3
6.	MC + DTEA	9.7 1.0	36.0 5.0	28	88.6 1.0	0.67 0.02	24.9

As demonstrated in Tab. 3 the almost complete release of drug from MCCh-hydrogel was observed. Two stage of the release of DTEA from MC and MCCh-hydrogels as well as DNa from MCCh-hydrogel has been observed (for another system only slower stage). DTEA from MCCh-hydrogel shows sustained-release properties as compared to commercial gel (Olfen). The presence of hydrophilizing agents (PG, G) increases the DNa release rate as compared to MC and MCCh-hydrogel without any hydrophilizing agents.

Kinetics release of zinc ions

Microcrystalline chitosan existing in the form of a gelatinous aqueous dispersion is characterized by several useful properties, such as a high water retention value, direct film-forming from a dispersion, as well as high metal chelating ability[7]. Microcrystalline chitosan based on its properties seems to be very effective material to prepare the chelates with metal ions having stimulating behaviour for wound healing.

The samples of microcrystalline chitosan chelate dispersion samples were subjected to prepare the suitable film. The metal ions release was realized by degradation of metal ion-chitosan chelating connection by water. The rate of zinc ions release from the hydrogel-systems through membrane was lower than that for the film-system immersed directly in a water bath (Fig. 3). On the other hand, the zinc ions release from aqueous solution trough membrane in primary time (30 min) was similar to hydrogel but then significantly greater and faster in comparison with the hydrogel. The above studies were confirmed the low release of zinc ions from microcrystalline matrix. The release process inspection realised in a current preliminary research was so interesting that its continuation should be necessary in future.

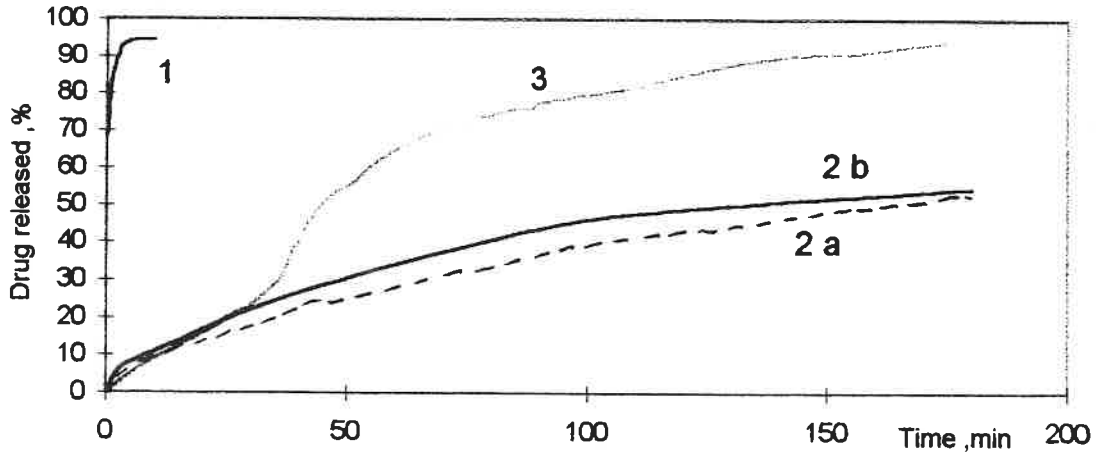

Fig. 3. Release profiles of Zn (II) ions from: MCCh-film (1), MCCh-hydrogel - determined by measurement of electric conductivity (2 a), MCCh-hydrogel - determined by UV spectroscopy (2 b) and dissolution profile of Zn (II) ions from aqueous solution (3)

Conclusion

On the ground of studies, it was found that the type of vehiculum has a great influence on the rate of active substance diffusion. The release depends on percentable content of MCCh in pharmaceutical phase. Beside MCCh vehiculum should be composed of closed definite amount of water, which is conductive to hydratation process of *stratum corneum* acting the function like the natural promotor (activator) of sorption.

The considerable amount of active substance was released at prolong time from chitosanous vehiculum, which contained the substance increasing the viscosity. This construction of vehiculum causes, on the one hand, the drying up of vehiculum (water evaporation from hydrogel structure), on the other, the forming of the hydrogel film on the skin surface, which is conductive to diffusion of healing substance through *stratum corneum.*

The investigations concerned the effective transportation of active substance through the membrane in vitro indicate that the presence of MCCh in multicomponent pharmaceutical phase causes the slowing down of its diffusion. Consequently the presence of MCCh in drug form ensures the control therapeutic efficiency.

The studies were confirmed the slow release of zinc ions from the microcrystalline chitosan matrix that can play an important role especially for medical use.

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NASAL DRUG DELIVERY WITH PROTASAN™

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Abstract

The nasal route of administration has advantages that allow the development of non-parenteral delivery systems for challenging drugs. The cationic bioadhesive polymer chitosan can have a dramatic effect in increasing the transport of polar drugs across epithelial surfaces [1].

Keywords: chitosan, chitosan glutamate, PROTASAN, nasal drug delivery, tight junctions, drug delivery, insulin.

Materials and methods

Chitosan glutamate was manufactured by Pronova Biopolymer as illustrated in the manufacturing scheme presented in Figure 1. The PROTASAN trademark covers several commercial grades of water-soluble chitosans for the pharmaceutical and biomedical industry. The materials are available as various salts of which chitosan chloride and chitosan glutamate are recommended. Each grade of PROTASAN is available as standard and ultrapure (UP) qualities.

Chitosan glutamate was made up in phosphate buffer. This was mixed with semisynthetic sodium insulin (Nova-Nordisk, Denmark) to obtain final concentrations of chitosan of 0.1%- 1.0% w/v. The insulin concentration was 200 IU/ml. pH was adjusted to 4.4 with HCl. The solutions were administered nasally (0.01 ml/kg) to groups of three or four cross-bred sheep (about 40 kg). Blood samples were taken from a jugular vein catheter. Plasma glucose levels were determined by the glucose oxidase method and the plasma insulin was determined by a double-antibody radioimmunoassay.

Safety of nasally administered chitosan glutamate was determined by

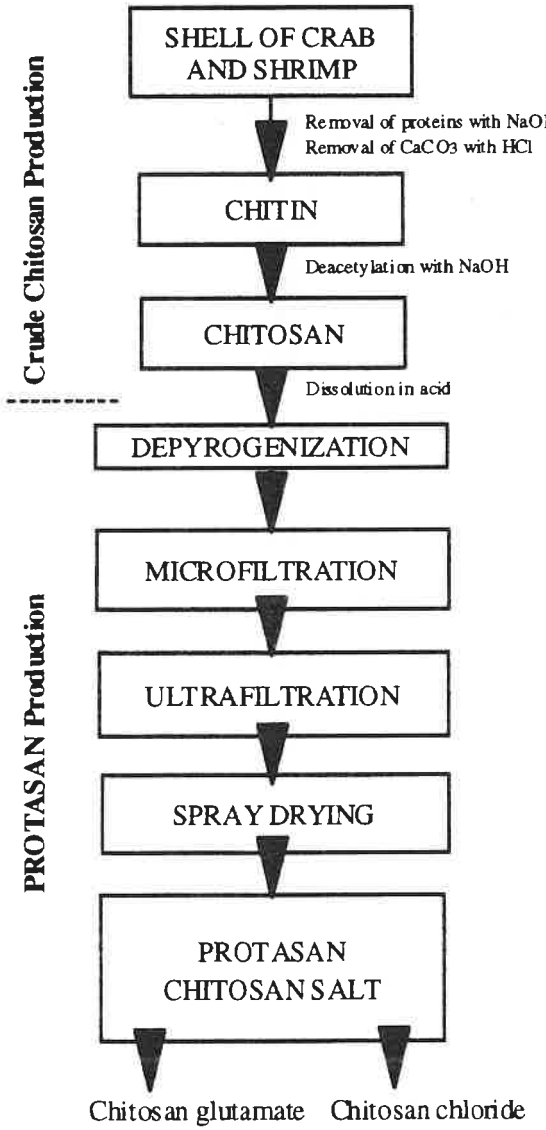

Figure 1: Manufacturing scheme for PROTASAN™ chitosan salts

treating rats with 0.5 or 1% chitosan glutamate solutions 3 times a day for 7 consecutive days. Histological sections of the nasal cavities were stained with hematoxylin and eosin.

Results and discussion

The effect of chitosan on the absorption of insulin from the nasal cavity in sheep is shown in Figure 2. Nasal administration of insulin as a simple phosphate buffer solution results in very low plasma concentrations of insulin. However, when chitosan glutamate was formulated together with insulin, the plasma insulin level increased rapidly reaching a peak at about 75 minutes following nasal administration of the combination.

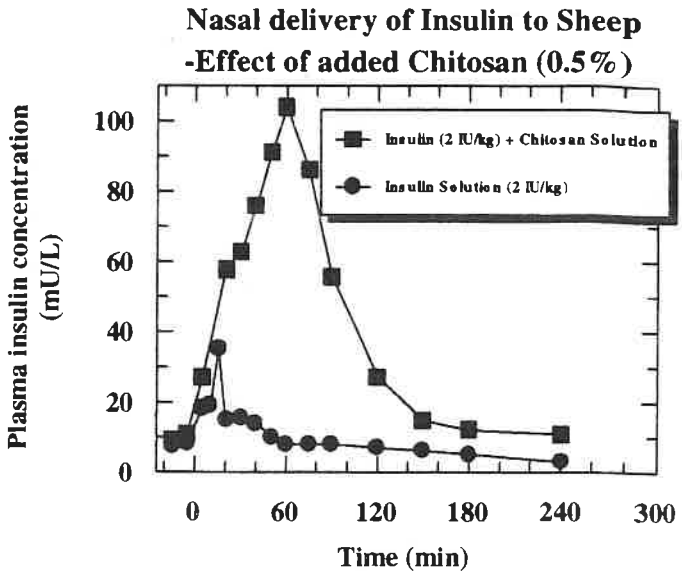

Figure 2: Nasal delivery of insulin to sheep: Effect of chitosan glutamate (0.5%)

Solution and powder formulations based on chitosans of a molecular weight of greater than 100 kD and with a defined degree of deacetylation, have found utility in improving the delivery of nasally administered polypeptides such as insulin. Figure 3 illustrates the increase in plasma insulin concentration when sheep were administered insulin nasally in combination with chitosan glutamate as a solution or as a powder formulation. As is apparent, the bioavailability of polypeptide drugs can be enhanced considerably. Similar results have been obtained in rats [2].

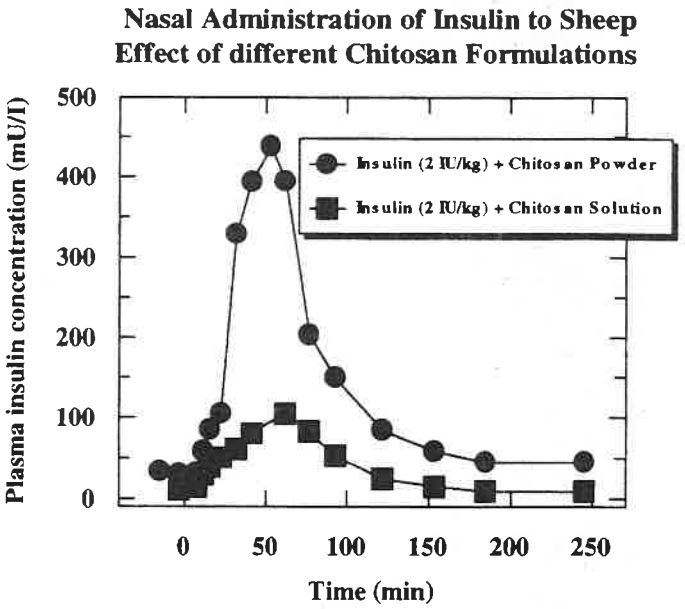

Figure 3: Effect of chitosan glutamate solution and powder on nasal absorption of insulin

Chitosan is believed to exert its effect by two mechanisms. The cationic polymer can bind to negative sialic residues in the mucus lining the nasal epithelial cells thereby slowing clearance of the formulation from the nasal cavity. Chitosan glutamate also has a direct effect on tight junctions between epithelial cells [3]. The tight junctions open transiently to allow an increased paracellular transport of polar molecules. Figure 4 shows the results from an *in vitro* study of the effect of chitosan glutamate on permeability of a confluent monolayer of CaCo-2 cells to mannitol. While mannitol permeability across the cell monolayer is quite low, the presence of chitosan glutamate increases this permeability. The effect appears to be pH sensitive, thus indicating that the charge-density of the chitosan molecule is of importance.

Detailed toxicological assessment in animals and man have shown that chitosan glutamate has no deleterious effects on the nasal mucosa nor on mucociliary transport [4,5]. An example of the transient chitosan-induced effects found in a nasal tolerance test in rats is shown in Figure 5. Here rats were administered 0.5 or 1% solutions of chitosan glutamate nasally three times a day for 7 consecutive days. There was an increase in mucus production in treated animals as compared to animals administered the vehicle (0.9% NaCl). However, the increase in mucus production was not dose-related. There was no toxic effect on ciliated cells, thereby confirming the integrity of cilia function and explaining the lack of effect on mucociliary transport.

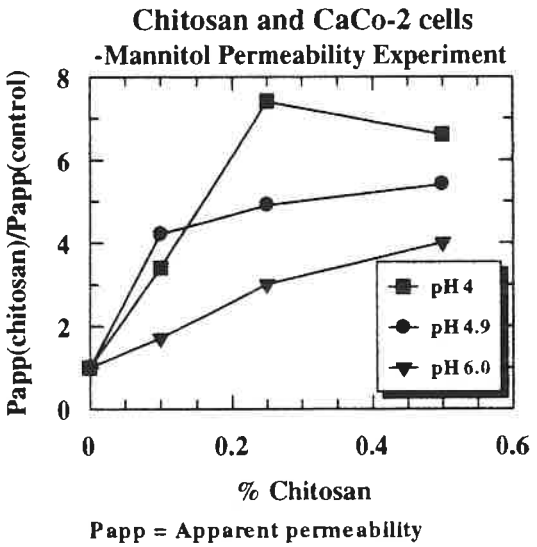

Figure 4: Effect of chitosan glutamate on CaCo-2 permeability

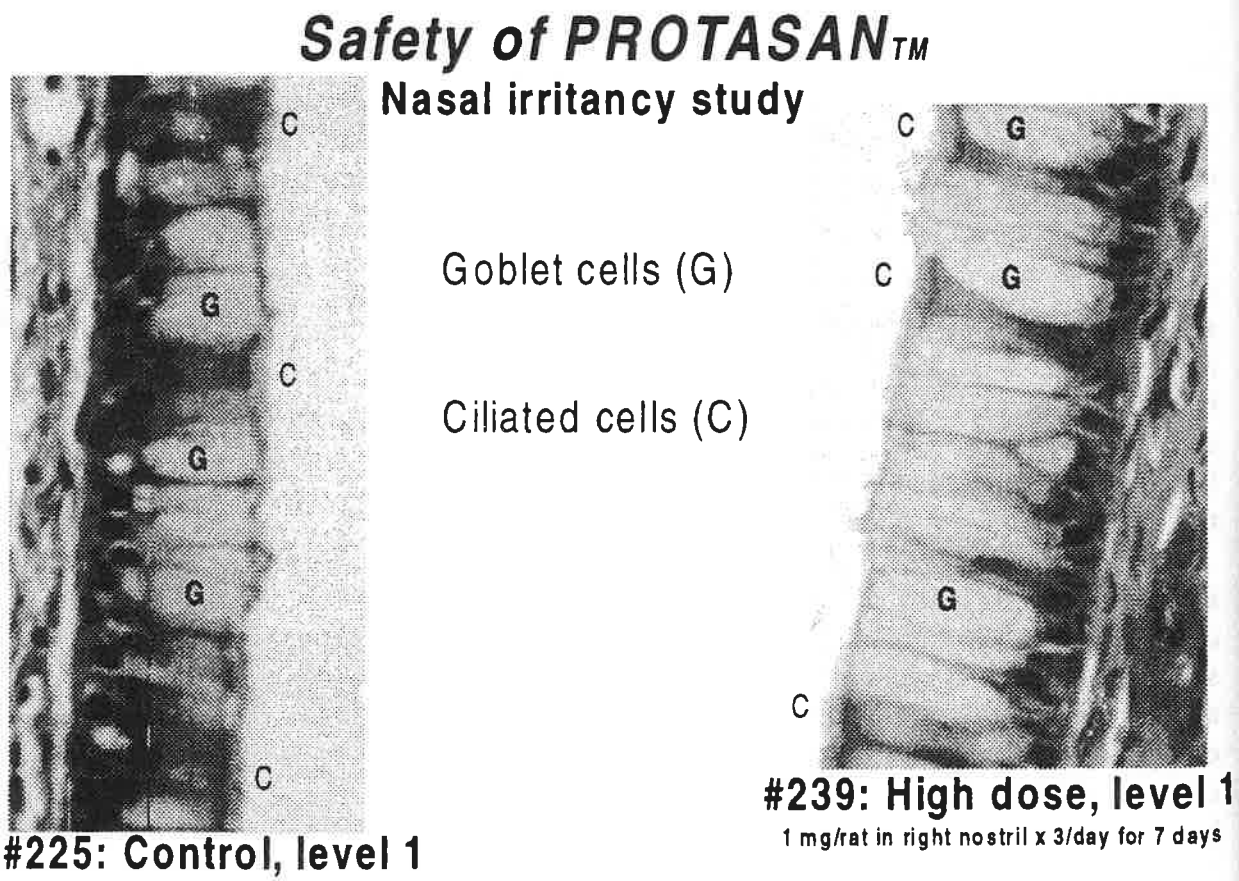

Figure 5: Effect of chitosan glutamate on rat nasal epithelia

Conclusion

When chitosan was introduced as an industrial product in the early 1970's, the field of wound healing played a significant role in the initial commercial development of the biopolymer. For new pharmaceutical and biomedical applications of chitosan to be successful, effect studies like the one presented here will be of importance. These studies illustrate that chitosan glutamate is able to increase the permeability of the nasal epithelium to the polypeptide drug insulin. The use of chitosan glutamate in a nasal formulation for drug delivery is appealing, and, as presented, the use of chitosan glutamate results in little or no toxicity to the nasal mucosa. Regulatory issues, however, will have to be addressed. In addition to characterization and functionality, the commercial manufacture of chitosan products for pharmaceutical use must also include product reproducibility. PROTASAN salts are manufactured under GMP guidelines to insure quality control and documentation for commercial grades of water-soluble chitosan salts. PROTASAN salts of chitosan show great potential in the development of drug formulations increasing the absorption of drugs across the nasal mucosa.

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