Growth modulation of fibroblasts by chitosan-polyvinyl pyrrolidone hydrogel: Implications for wound management?

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Wounds in adults and fetuses differ in their healing ability with respect to scar formation. In adults, wounds lacking the epidermis exhibit excess collagen production and scar formation. Fibroblasts synthesize and deposit a collagen rich extracellular matrix. The early migration and proliferation of fibroblasts in the wound area is implicated in wound scarring. We have synthesized a hydrogel from chitosan-polyvinyl pyrrolidone (PVP) and examined its effect on fibroblast growth modulation *in vitro*. The hydrogel was found to be hydrophilic as seen from its octane contact angle (141·2 ± 0·37°). The hydrogel was non-toxic and biocompatible with fibroblasts and epithelial cells as confirmed by the 3(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) assay. It showed dual properties by supporting growth of epithelial cells (SiHa) and selectively inhibiting fibroblast (NIH3T3) growth. Growth inhibition of fibroblasts resulted from their inability to attach on to the hydrogel. These findings are supported by image analysis, which revealed a significant difference ($P < 0·05$) between the number of fibroblasts attached to the hydrogel in tissue culture as compared to tissue culture treated polystyrene (TCPS) controls. However, no significant difference was observed ($P > 0·05$) in the number of epithelial (SiHa) cells attached on to the hydrogel as compared to the TCPS control. Although *in vivo* experiments are awaited, these findings point to the possible use of chitosan-PVP hydrogels in wound-management.

1. Introduction

Wound healing is an active area of interest for many researchers on account of its importance in the treatment of burns, prevention of post surgical adhesions and cosmetic surgery. Full-thickness wounds i.e., wounds involving damage to both epidermis and dermis, heal by epithelialization, wound contraction and scarring. In adults, unhealed burn wounds, which lack the epidermis, demonstrate excess collagen production and scar formation. The fibroblast is a cell type, which synthesizes and deposits a collagen-rich extracellular matrix. Early migration of fibroblasts and their proliferation in the wound area is implicated in wound scarring (Jobson *et al* 1998). Tissue fibrosis also manifests itself in the form of post-surgical adhesions which have been identified as a common problem contributing to the failure of gastrointestinal, gynecological and sinus surgeries (Schippers *et al* 1998; Ramadan 1999). Given the desirability of wound healing without scar formation, clinical strategies to decrease scar formation should thus attempt to achieve early wound closure with synthetic epidermal substitutes, skin grafting or the application of cultured epithelial autografts (Horch *et al* 1998). Synthetic epidermal substitutes with cultured epithelial cells seem to be an attractive strategy (Compton *et al* 1998) since keratinocytes have been demonstrated to modulate fibroblast growth and collagen synthesis (Garner 1998).

Many materials have been tested for wound management applications. Some of these materials have demonstrated reduced fibrosis and accelerated wound healing (Minami *et al* 1993). One of the most important criteria to select materials for wound management, is their biocompatibility. According to the modern definition, a biocompatible material need not be inert. But it should be bioactive in terms of its appropriate reactivity for the intended

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application. Non-biocompatible materials are reported to elicit inflammatory reactions which severely limit their use (Ekholm *et al* 1999).

Interestingly, fetal wounds heal without fibrosis or scar formation (Lorenz and Adzick 1993). The mechanisms underlying this remarkable process are mediated in part through the fetal wound extracellular matrix, which is rich in hyaluronic acid. Prolonged presence of hyaluronic acid in the matrix of fetal wounds creates an environment that promotes fetal fibroblast movement (Longaker *et al* 1991). Chitosan, a partially deacytylated product of chitin, is composed of D-glucosamine, which is also present in disaccharide subunits of hyaluronic acid. Chitosan thus structurally mimics hyaluronic acid and could exert similar effects. Chitosan has been reported to be bioabsorbable and biocompatible (Muzzarelli *et al* 1988). It has also been used in wound dressing matrices (Jayasree *et al* 1995) and is shown to be a wound healing accelerator (Minami *et al* 1993). Polyvinyl pyrrolidone (PVP) is a synthetic polymer that has been shown to be biocompatible; UV-cured films of N-vinyl pyrrolidone copolymers have been proposed as a potential bioadhesive wound dressing matrix (Kao *et al* 1997). Due to its lubricity and viscous properties, PVP has been used to coat tissue contacting surfaces (Howard 1988) and as vitreous humor substitute (Hong *et al* 1998).

Risbud *et al* (1999) have synthesized chitosan–PVP hydrogels and demonstrated their suitable properties and *in vitro* biocompatibility for biomedical applications. However, there is no report on this hydrogel's ability to modulate fibroblast growth. We report here *in vitro* fibroblast growth modulation by a chitosan-PVP hydrogel, and suggest that the phenomenon may prove to be of use in wound management.

2. Materials and methods

2.1 *Materials*

Chitosan (deacytylation degree > 80%) and PVP were purchased from Vishu Aquatech, Chennai, and SRL, Mumbai, respectively. Glutaraldehyde was purchased from ICN Biomedical, Ohio, USA. NIH3T3 (ATCC CRL-1658) and SiHa (ATCC, HTB-35) cell lines were obtained from NCCS cell repository. All other chemicals or reagents used were of analytical grade.

2.2 *Hydrogel synthesis*

The hydrogel was synthesized as earlier reported (Risbud *et al* 1999). In brief, a chitosan solution 2% (w/v) was prepared by dissolving chitosan in an aqueous solution of acetic acid (0·1 N). PVP solution 4% (w/v) was prepared in double distilled water. A final mixture (1 : 1 v/v) of chitosan and PVP was prepared. Glutaraldehyde (25% aqueous solution) was added with continuous stirring at 0·1% v/v of chitosan-PVP polymer solution. This solution was poured in 35 mm tissue culture dishes (Nunclone, Denmark) to coat the dishes and kept for drying at 30 ± 2°C for 48 h in a sterile atmosphere. Prior to their use, the hydrogel coated dishes were washed extensively with PBS (pH 7·4) to remove any unreacted glutaraldehyde. Tissue culture treated polystyrene (TCPS) dishes coated with chitosan alone and without any coating served as controls.

2.3 *Octane contact angle determination*

The octane contact angle method was employed to quantify polar interactions across the polymer-water interface (Bagnall *et al* 1980). This method is suited for the quantitative assessment of surface hydrophilicity of hydrophilic surfaces as they are fully hydrated. Hydrogels were mounted on microscope slides and supported in an inverted fashion in a container. The container was filled with double distilled water to immerse the slide. A goniometer (Kernco Instruments Co. Inc., Texas, USA) was aligned and focused on the polymer water interface. A micro syringe containing 99·99% n-octane was lowered in to the water and a drop of around 0·1 µl was introduced at the polymer water interface. Contact angles on both sides of the drop were immediately measured, assuming symmetry. The present data represent the mean ± SE of at least 25 contact angles per membrane.

2.4 *Determination of hydrogel cytotoxicity by MTT assay*

Sterile hydrogel pieces of uniform weight (50 mg each) were incubated in 10 ml of Dulbecco's modified Eagle's medium (DMEM)/minimum essential medium (MEM) at 37°C for 15 days. This medium, containing the membrane leach-out products was then filtered through a 0·22 µ filter and used at various concentrations for testing the effect of leach-out products on cellular viability. NIH3T3 and SiHa cells were seeded in 96 well plates and incubated for 24 h at 37°C in 5% CO_2, 95% air. Cells were then exposed to varying concentrations (10–30%) of hydrogel leach-outs for 24 h. The MTT assay was performed according to standard protocol (Doyle *et al* 1995) to assess cellular viability.

2.5 *Cultures of NIH3T3 fibroblast and SiHa epithelial cells on hydrogel*

NIH3T3 fibroblasts and SiHa cells were suspended in DMEM (Sigma, St. Louis, MO, USA) and MEM (Sigma, St. Louis, MO, USA) with 10% fetal calf serum (FCS, Gibco BRL, Gaithesburg, USA) respectively and seeded at a concentration of 2×10^5 cells/ml on chitosan-PVP hydrogel coated tissue culture dishes (35 mm, Nunclone, Denmark). Cells seeded on uncoated TCPS dishes and TCPS dishes coated with chitosan alone served as controls. Dishes were

maintained at 37°C in a humidified atmosphere with 5% CO_2 for 5 days. The culture medium was replaced with fresh medium every 48 h. Cultures were observed every day under an inverted phase contrast microscope (IMT 2, Olympus, Japan).

2.6 *Image analysis*

Cell numbers in control and hydrogel coated dishes were quantified using a digital image analysis system (DIA, Kontron Elektronik GmbH, Munchen, Germany) connected to a Zeiss Axioplan 2 microscope. Cells were observed and images captured with a VarioCam PCO CCD camera. Images were then processed to obtain binary images. Binary images were used to quantify cell numbers using a Kontron image analysis software (KS 400, V 2·0). Cells were measured from 50 different fields of area 712 × 512 pixel on each dish. The mean cell number present on TCPS controls at each time point is considered as 100%.

2.7 *Statistical analysis*

Results are expressed as means ± SE. Differences between groups were tested by Student's *t* test or the Mann Whitney test whichever was appropriate. Computations were performed using a Sigma-Stat statistical package (Jandel Scientific, version 2·0 for Windows 95, SPSS Inc., Chicago, USA).

3. Results

3.1 *Octane contact angle determination*

The octane contact angle of the hydrogel was found to be 141·2 ± 0·37° as compared to 136·2 ± 0·27° of unmodified chitosan.

3.2 *Hydrogel cytotoxicity determination*

Cytotoxicity of the hydrogel as determined by the MTT assay is shown in figure 1. NIH3T3 and SiHa cells were seen to proliferate (indicated by high per cent viability over control) when they were exposed to increasing concentrations of leach-outs of the chitosan-PVP hydrogel. Differences in per cent viability of SiHa and NIH3T3 cells could be ascribed to variation between cell lines.

3.3 *Fibroblast growth modulation by the hydrogel*

Microscopic observation of the fibroblast cultures revealed an interesting phenomenon. Initially (day 1 of culture) the major population of fibroblasts failed to attach on to the hydrogel. The cells showed a rounded morphology due to failure of attachment (figure 2a). However, the fibroblast showed perfect attachment on to the TCPS control (figure 2b). By day 3 of culture, those fibroblasts, which were attached on to the hydrogel showed spreading (figure 2c) and started to increase in number. During this period fibroblast on the TCPS control became almost confluent (figure 2d).

3.4 *Culture of epithelial cells on hydrogel*

From day 1 till day 5 of culture SiHa cells showed proper attachment and growth on the hydrogel (figure 3b, e) which was comparable to TCPS controls (figure 3a, d). Cell growth on an unmodified chitosan control was seen to be slower than that on both TCPS and the chitosan-PVP hydrogel (figure 3c, f).

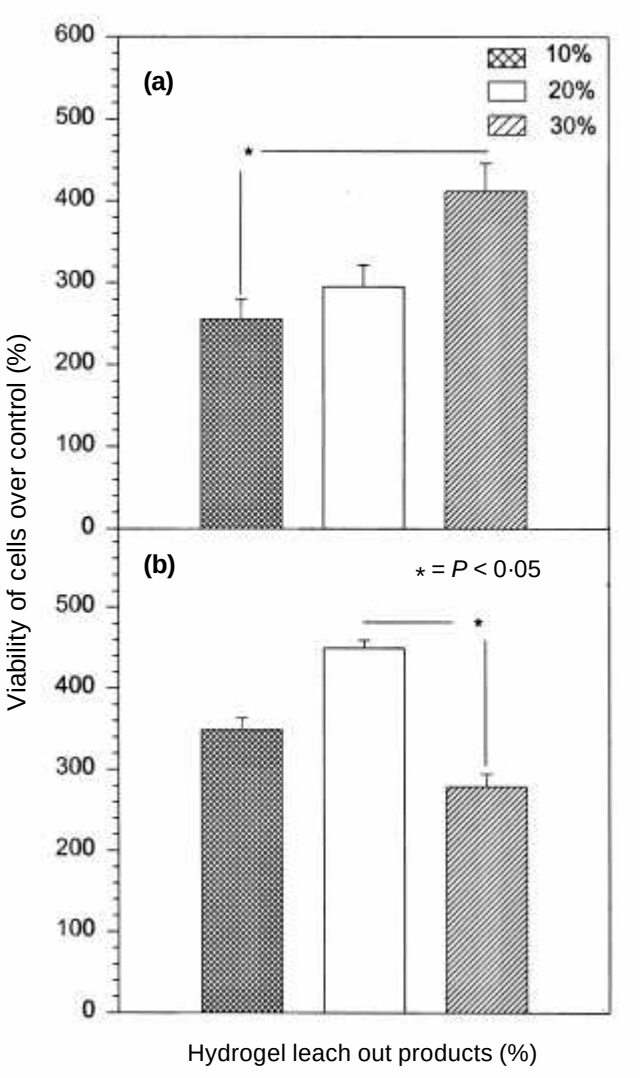

Figure 1. Per cent viability of (**a**) SiHa cells and (**b**) NIH3T3 fibroblats on exposure to hydrogel leach out products in varying concentrations as compared to unexposed control by MTT assay. Per cent viability of cells unexposed to leach out products is taken as 100. Note that per cent viability above 100 is indicative of cellular proliferation (*$P < 0·05$)

3.5 *Image analysis*

Image analysis studies (figure 4) reveal that there is a significant difference in the number of fibroblasts attached on the hydrogels as compared to TCPS controls ($P < 0{\cdot}05$). The number of fibroblasts attached on to the hydrogels was around 8% on day 1 and 45% on day 5 as compared to TCPS controls (at each time point number of fibroblasts on the TCPS control was considered as 100%). The number of SiHa cells on the hydrogels was slightly lower than cells attached on TCPS control on day 1. However, there was no significant difference in the number of SiHa cells attached on the hydrogel as compared to the TCPS control until day 5 of culture (figure 4).

4. Discussion

We have chosen chitosan-PVP hydrogels as a candidate for this study in view of the reported properties of chitosan in preventing fibroblast growth (Schmidt *et al* 1993) and the hydrogel's cytocompatible (Risbud *et al* 1999) and blood biocompatible (Risbud and Bhat 1999) nature. Addition of PVP to chitosan results in hydrogels with superior properties to unmodified chitosan in respect to mechanical strength, hydrophilicity and water content (Risbud and Bhat 1999).

The octane contact angle of a material is directly proportional to the surface hydrophilicity (Paul *et al* 1995) which, along with surface free energy, is correlated to protein adsorption on the surface (Walton *et al* 1997). Since proteins play a major role in cell adherence to substrata, surfaces that resist protein adsorption are likely to resist cell adhesion (Kenedi 1980; Lhommeau *et al* 1997). Chitosan-PVP hydrogels were seen to be highly hydrophilic as determined by the octane contact angle. Their increased hydrophilicity over unmodified chitosan is due to the presence of PVP, which is known to be highly hydrophilic (Howard 1988). Though more hydrophilic than unmodified

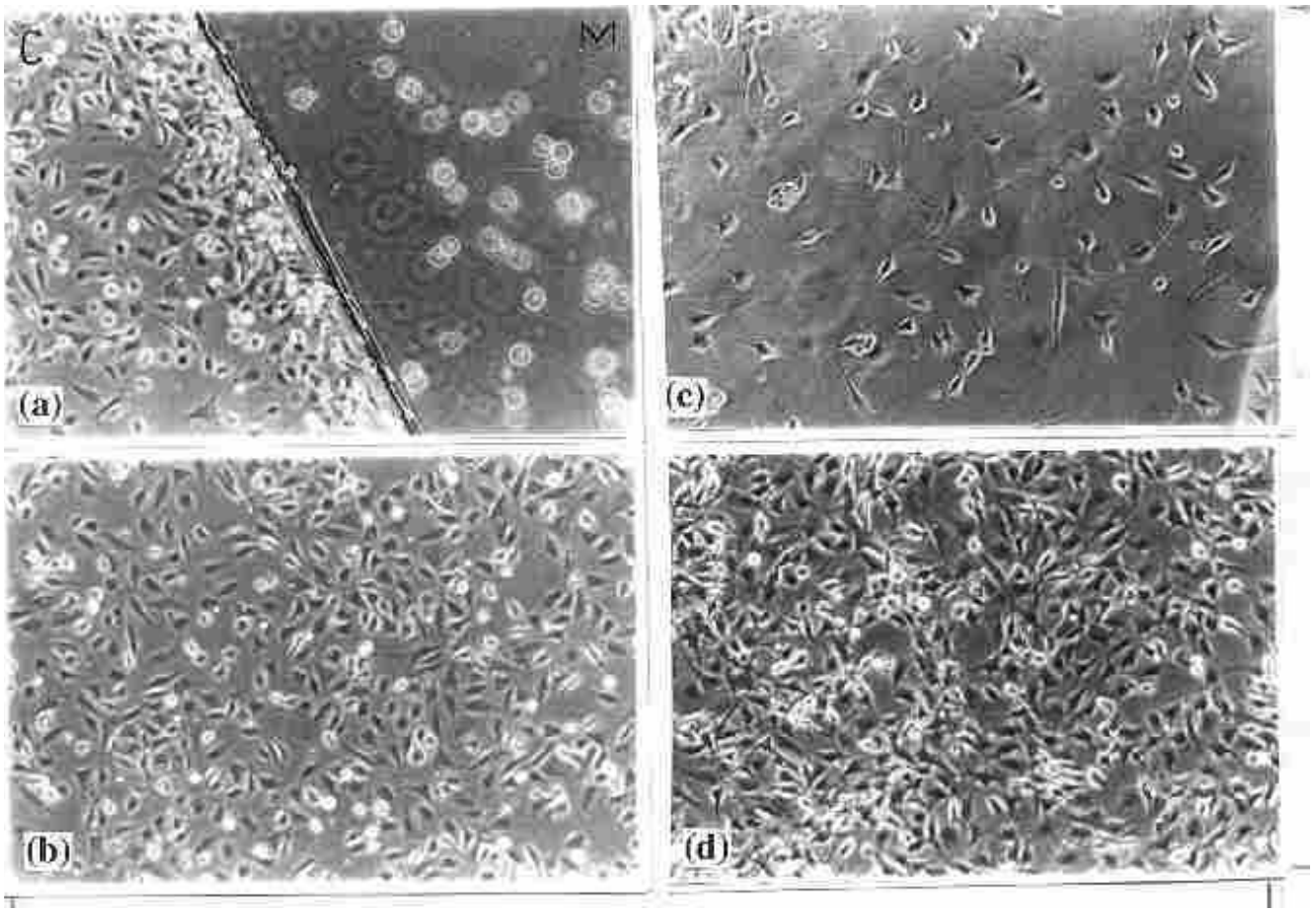

Figure 3. Growth modulation of fibroblasts by chitosan-PVP hydrogels. (**a**) Day 1 fibroblasts growing on TCPS control. (**b**) Day 1 fibroblasts start attaching on the hydrogel. (**c**) Day 3 fibroblasts attached on TCPS control. (**d**) Day 3 fibroblasts growing on the hydrogel. (× 300). C, Control; M, chitosan-PVP hydrogel.

Figure 4. Day 1 (**a**) SiHa cells growing on TCPS control. (**b**) SiHa cells growing on hydrogel. Note proper attachment and spread morphology of cells. (**c**) SiHa cells growing on chitosan. Day 3 (**d**) growth of SiHa cells on TCPS control. (**e**) Growth of SiHa cells on hydrogel. Note comparable TCPS control. (**c**) Day 3 (**f**) SiHa cells growing on the hydrogel (× 300). Day 3: fibroblasts growing on TCPS control (× 300). C, Control; M, chitosan-PVP hydrogel.

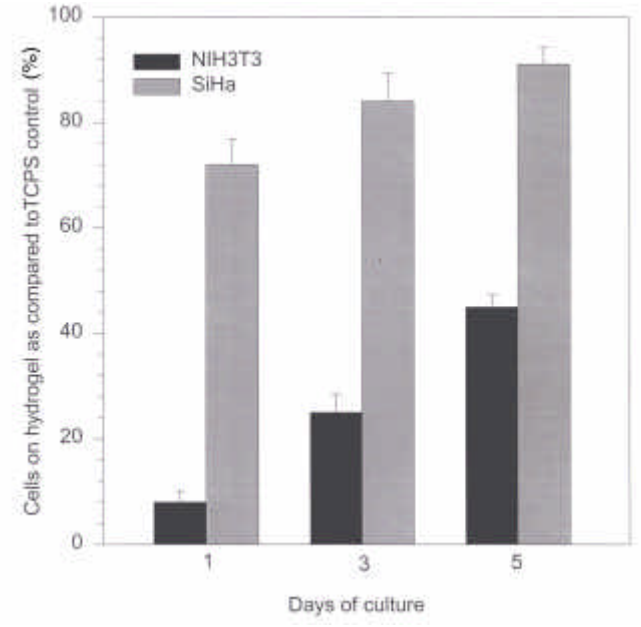

Figure 4. Image analysis studies showing growth profile of NIH3T3 and SiHa cells on chitosan-PVP hydrogel. Figure shows per cent of cells attached on hydrogel as compared to cells on TCPS control. The number of cells attached on to the TCPS control at each time point is considered as 100%.

chitosan the hydrogel is better suited for epithelial cell growth as PVP is known to support cell growth (Hong *et al* 1997). The chitosan-PVP hydrogels have been shown to resist protein adsorption on the surface (Risbud *et al* 1999). Thus, among the probable causes for decreased fibroblast adhesion on the chitosan/PVP hydrogel could be reduced protein adsorption.

As determined by the MTT assay, the proliferation (per cent viability > 100) of NIH3T3 and SiHa cells on exposure to hydrogel leach-out products (figure 1a, b), indicates the biocompatible and non-toxic nature of hydrogel confirming earlier reports about the biocompatibility of chitosan-PVP hydrogels (Risbud *et al* 1999). This could be due to leaching out of PVP from the hydrogel network, which has been shown to support cellular proliferation (Hong *et al* 1997). In the same study, during the cytotoxic evaluation of crosslinked PVP gels by a sulforhodamine B colorimetric assay, it was found that many of the gels showed protective/growth promoting effects on 3T3 mouse fibroblasts in static cultures (Hong *et al* 1997).

Chitosan-PVP hydrogels have exhibited differential growth supportive properties for epithelial and fibroblast cells. These hydrogels strongly inhibited fibroblast growth and proliferation by preventing their attachment on the surface (figure 2a). However, they supported attachment and growth of epithelial (SiHa) cells (figure 3b, e) in a manner comparable to that shown with TCPS control. Pure chitosan controls showed decreased epithelial cell growth than both TCPS and hydrogel (figure 3c, f). Enhanced growth of epithelial cells on hydrogel than chitosan alone could thus be attributed to the presence of PVP. Observations about modulation of cell growth by hydrogel are also supported by image analysis, which clearly demonstrate that there is a significant reduction ($P < 0{\cdot}05$) in number of fibroblasts attached on the hydrogel than on the TCPS control (figure 4). However, no significant decrease ($P > 0{\cdot}05$) was observed in number of epithelial cells attached on hydrogel as compared to TCPS control (figure 4). There are several reports about effect of chitosan on fibroblast growth. Inability of fibroblast to attach and proliferate in the presence of chitosan has been reported (Malette *et al* 1986; Schmidt *et al* 1993). Mori *et al* (1997) reported deceleratory effect of chitin and its derivatives on the proliferation of cultured L929 fibroblasts. Chitosan coated alginate microcapsules have been shown to arrest capsular fibroblast growth *in vivo* (Hardikar *et al* 1999). However, chitosan matrices have been demonstrated to support attachment and growth of other cell types such as hepatocytes and adrenal chromaffin cells (Kawase *et al* 1997; Eser Elcin *et al* 1998). Thus, the present results are in agreement with the earlier reports. The properties of chitosan-PVP hydrogel thus mimic fetal wound healing as it selectively inhibits fibroblast growth and promotes epithelial cell growth, which is otherwise deficient in adult wounds.

Wounds and burns are susceptible to infections by bacteria and fungi. Infections lead to delayed wound healing, septicaemia and a wide spectrum of other complications. Accumulation and migration of immune cells like macrophages and neutrophils in the wound bed is thus necessary for body's defence against such external infections. Wounds applied with chitosan show accumulation of polymorphonuclear cells, angiogenesis and healing with minimum scarring (Usami *et al* 1997). Chitosan is also known to possess excellent antifungal and antibacterial property (Allan *et al* 1979; Nelson *et al* 1994). Thus these properties could be an added advantage for chitosan blended material to be used in hostile environments of wounds and burns.

The present study clearly demonstrates the growth modulating effects of chitosan/PVP hydrogel on fibroblasts *in vitro*. An attractive possibility is that it could be used in wound management products to check excessive fibrosis.

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