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The effect of antibacterial agents on the behaviour of cultured mammalian fibroblasts

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Wound infections retard healing rates and can lengthen the time a patient is required to spend in hospital. Obviously it is desirable to minimize or remove pathogenic organisms to allow wound healing to progress optimally. At present there are a large range of topical applications available in the form of antibiotics or antiseptics, but the toxic effects of these agents may outweigh the benefits. In this study two conventional antiseptic agents were examined together with a range of phosphate-based glasses which were designed to release metal ions into an aqueous environment. As a control a series of standard metal ion solutions were prepared and examined in the same test system. The metal ions examined are considered important in the wound healing environment either for their potential bacteriostatic effects or indeed their potential for increasing cellular activity. This part of the study examined the effects of these agents on mammalian cells *in vitro*, using a standard MTT assay to assess the cytotoxicity or activity of cultured mouse fibroblasts. It was found that chlorhexidene, a commonly used antiseptic agent, was toxic at low levels. The controlled release glasses, in most cases, increased the rate of cell activity before reaching toxic levels. This effect was also found with the standard metal solutions.

1. Introduction

In the effort to combat infection at wound sites, antibacterial agents have been incorporated into a variety of wound dressings. A number of agents have proved effective in their ability to inhibit bacterial or fungal growth. However, some of these agents have been shown to have a deleterious effect in the delicate environment of a healing wound and may retard the rate of wound healing.

The first antiseptic wound cleansing agent to be used extensively in the First World War was Dakin's solution (surgical chlorinated soda solution, BPC 1973). It had a dramatic effect in reducing wound sepsis, but to be effective the wounds had to be irrigated with large volumes of the solution. Cleansing by washing out with antiseptics can neutralize naturally occurring wound hormones or debriding proteases which are part of the natural defence mechanism. Barton and Barton [1] suggested that in the treatment of pressure sores the use of hypochlorites, like other topical antiseptics and antibiotics, can cause bacteria present in the wound to release toxic materials and produce acute oliguric renal failure. Studies into the effect of antiseptics and topical antibacterial agents on wound healing by Leaper and Simpson [2] have shown that long-term use may delay healing. It was suggested that the use of hypochlorite solutions could lengthen a patient's stay in hospital and thus the length of time required to resume their normal life.

Another available range of antiseptics contain the potent antimicrobial agent povidone-iodine, but with all preparations containing iodine there is the possibility of adverse reactions in some patients [3]. It has been reported that iodine-containing antiseptic agents cause thyroid dysfunction in very low birthweight babies, thus leading to hypothyroidism during a critical period of their development.

A series of *in vivo* studies in which the antiseptics eusol and chloramine were introduced into a rabbit ear chamber preparation showed that they permanently disrupted capillary circulation in granulation tissue and delayed the healing process [4]. Kozal *et al.* [5] investigated the effects of dilute solutions of sodium hypochlorite (Dakin's solution) on the viability of neutrophils, fibroblasts and endothelial cells. It was shown that hypochlorite solutions with concentrations of just 0.0025% caused cell damage. A further study [6] looked at the effect of 0.0002% hypochlorite solutions on the random migration of neutrophils against a known chemotactic agent. The movement of neutrophils was hindered by this concentration of hypochlorite solution. When the morphology of the cells was studied with electron microscopy it was unchanged, indicating no structural cell damage. Therefore it was concluded that the normal functions of the neutrophils had been inhibited. As neutrophils and macrophages play an important part in the removal of debris and bacteria at a wound site, the wound healing process could be retarded.

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Silver and copper are known to have useful biocidal properties. For many years silver salts have been used successfully in creams for topical application to wounds. This is particularly useful as the application of antibiotics in this manner can potentially induce an allergic sensitivity reaction and may lead to the development of resistant strains of bacteria. Furthermore, silver and its compounds are being widely investigated for use in medical devices such as urinary and intravascular catheters. All copper compounds attack mucous membranes even in low concentrations and thus have been used as superficial antiseptics. Copper compounds have been used as pesticides and fungicides in horticulture and farming.

Phosphate-based glasses, which are soluble in aqueous environments, have been shown to be biocompatible [7] and it is possible to incorporate metal ions into the glasses such that a steady release of ions can be provided at the wound site. Unlike chemical compounds which are stoichiometric and have a unique set of properties, it is possible to change the composition of the controlled release glass to vary properties such as the solution rate and the pH of the resultant solution. The physical properties of the glasses are affected by temperature and the pH of the medium in which they are immersed. The solution rate is increased two-fold by a rise in temperature of 10 °C and is kept at a minimum when the pH is 7.

Controlled release glasses (CRG) and conventional glasses are similar in physical characteristics such as transparency and non-crystallinity; however, the glass network former of CRGs consists of phosphates or borates rather than silicates. The glasses are organic compounds that are prepared in a stable glassy form which dissolve in an aqueous environment at any predetermined rate, and it is possible to incorporate any inorganic element into the structure of the glass. As the solution is controlled by the chemical composition of the glass, it is impossible for the controlled release process to breakdown and lead to a potentially dangerous situation where there is a large influx of metal ions or cessation of their release.

This study examines the potential for CRGs in the wound environment to provide a release of bacteriostatic or antiseptic agents. The objectives of this part of an ongoing study are to determine the levels at which these released agents have an effect on cultured mammalian fibroblasts.

In the study the *in vitro* cytotoxic effect of various antibacterial agents is measured by means of mammalian cell culture using an MTT assay [8]. For comparison a range of standard metal ion solutions and cell culture mediums were employed as controls.

The other materials used for comparison in this study are all well-known commercially available antiseptic agents which are currently used in wound cleansing in the form of topical creams, powders or solutions.

2. Materials and methods

The materials used in this study were CRGs supplied by Giltech Ltd, Ayr, UK, containing silver, copper, magnesium and zinc ions, chlorhexidine diacetate salt, iodoform (0.9 wt % iodine) and polyvinylpyrrolidone iodine complex with 11.4% available iodine (all from Sigma Chemical Co., USA). The compositions of the CRGs are shown in Table I.

It has been shown that the biocidal effects of silver, copper and iodine occur at levels of 10, 110 and 200 p.p.b., respectively [9]. A range of exudates/solutions were prepared at concentrations of 1, 10, 100, 250, 500 and 1000 p.p.b. using sterile distilled water and cell culture medium. The standard metal ion solutions were diluted to 2000 p.p.b. from stock solutions of 1000 p.p.m. by adding 1 ml to 500 ml of sterile distilled water. The antiseptics and CRGs were presented as powders; the weight of each was calculated using simple mathematical techniques to give a solution containing 2000 p.p.b. Solutions of each material containing 2000 p.p.b. were diluted with X2 culture medium to 1000 p.p.b. From this stock solution the dilutions were made with X1 culture medium. The control medium was a solution of 50% X2 cell culture medium and 50% sterile PBS.

Microtitre plates with 96 wells were used throughout the study; 12 wells were used as the number of samples in each group.

L929 mouse fibroblasts (obtained from ICN Flow, UK) were used for the procedure. They were maintained in X1 Modified Earles Medium (MEM) culture medium with 5% fetal calf serum. The cells were incubated at 37 °C in a 5% CO_2/95% air atmosphere, grown to confluence in a flat bottomed flask, and the monolayer harvested by trypsinization. The cells were subcultured following a routine procedure and seeded in the microtitre plates with 200 µl of 10^5 cell suspension per well. The plates were then re-incubated for 48 h. After this time the medium was removed and replaced with the prepared exudates/solutions.

The plates were then incubated for 24, 48 and 72 h, after which the medium was removed. A total of 100 µl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution, at a concentration of 5 mg ml^{-1}, was added to each well and the plate incubated for 4 h at 37 °C. After this period the solution was removed and replaced with 50 µl of isopropanol. The plates were again incubated for a further 20 min and then gently shaken to ensure complete dissolution of the blue formazan crystals. The optical densities of the solutions were measured by an enzyme-linked immunosorbent assay (ELISA) plate reader at a 570 nm test wavelength.

TABLE I The compositions of the controlled release glasses. The glasses used for this study are slow releasing and are designed to be used in a powder form which was appropriate for this procedure. The amount of glass which was used contained metal ions at a level of 1000 p.p.b.

Oxides	CRG–Ag (mol %)	CRG–Cu,–Mg,–Zn (mol %)
Na_2O	20	20
CaO	29	27
Ag, Cu, Mg, Zn	3	5
P_2O_5	48	48

3. Results and discussion

Chlorhexidine showed the greatest cytotoxic effect after 24 h (Fig. 1). It has been reported in previous work that tissue culture results show chlorhexidine is relatively free of toxicity; this was also found *in vivo* by Brennan *et al.* [10]. The other materials had a deleterious effect on cultured cells at levels of 500 p.p.b. After 24 h it was seen that the copper-releasing glass induced a cytotoxic effect above 250 p.p.b. For copper to be useful in combating bacteria and fungi it is required to be present at a concentration of 110 p.p.b. The copper-releasing glass only showed toxicity at 48 and 72 h at a concentration of 1000 p.p.b. (Fig. 2).

Silver ions, which have been shown to be biocidal at a level of 10 p.p.b., had little effect up to a concentration of 1000 p.p.b. (Fig. 3). After 48 and 72 h copper seems to have stimulated the growth of cells (Fig. 2), whereas this was not seen with silver ions (Fig. 3).

The phenomena which occurred with the copper-releasing glass was also seen with the magnesium- and zinc-releasing glasses. The magnesium and zinc glasses caused the cells to behave in a similar fashion, only becoming toxic at 1000 p.p.b. after 24 and 48 h (Figs 4 and 5). The effect on the cells at 72 h was that the magnesium glass encouraged a greater rate of cell proliferation than the zinc glass, except at 250 p.p.b.; at this concentration there is no difference between the effect each glass has on the cultured cells. All glasses apparently have the effect of increasing the metabolic rate of the cells after 48 h at concentrations of 10–500 p.p.b., the effect being more pronounced with the copper-releasing glass after 72 h.

The antiseptic agent polyvinylpyrrolidone (PVP) became toxic at 1000 p.p.b. for all time periods. Below this concentration there was no detrimental effect on the growth of cells. Between 10 and 500 p.p.b. the proliferation of the cells seems to have been increased by the presence of PVP, indicating that the cells can tolerate this particular antiseptic (Fig. 6).

There is a clear trend observed with PVP, CRG with magnesium and zinc ions and to a lesser extent with silver ions (Figs 3, 4, 5 and 6) where at lower concentrations cell growth is seen to increase with respect to the controls. As the concentrations increase

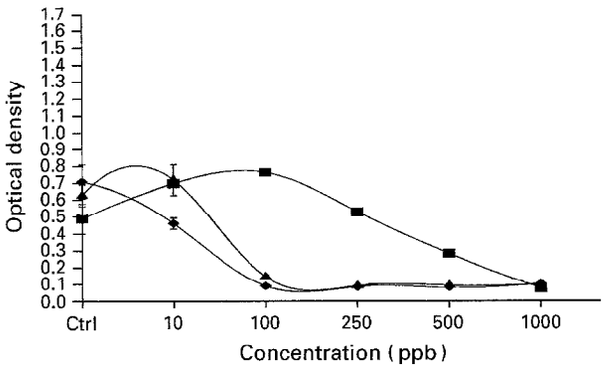

Figure 1 MTT assay with chlorhexidine. This was the only agent to decrease cell activity after 24 h.

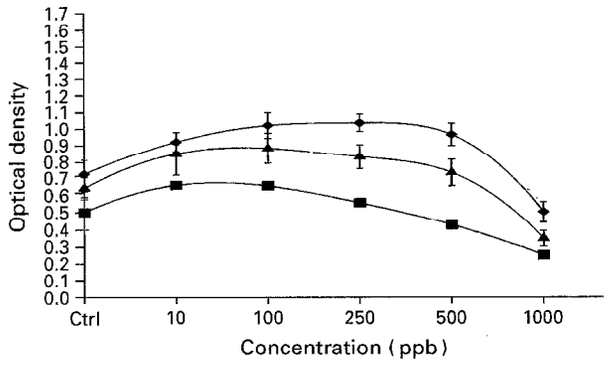

Figure 2 MTT assay with CRG/5 mol % Cu. At lower concentrations the copper-releasing glass seemed to have the effect of increasing the rate of cell metabolism.

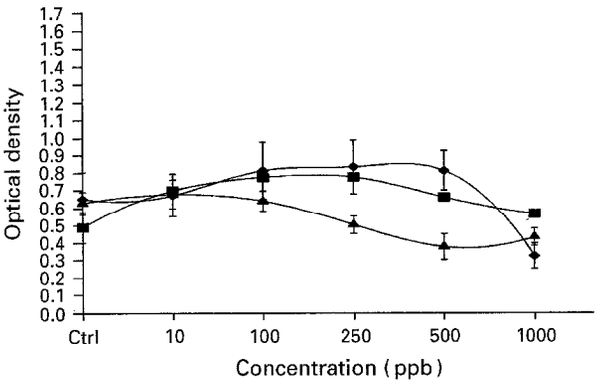

Figure 3 MTT assay with CRG/3.5 mol % Ag. After 24 h the silver-releasing glass does not seem to have any cytotoxic effect on the fibroblasts.

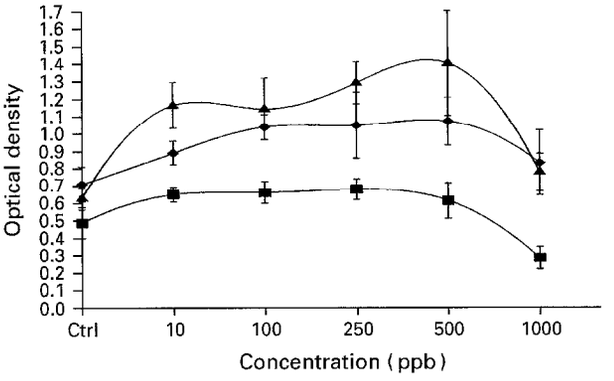

Figure 4 MTT assay with CRG/5 mol % Mg. The magnesium-releasing glass became cytotoxic at 1000 p.p.b. after 24 h.

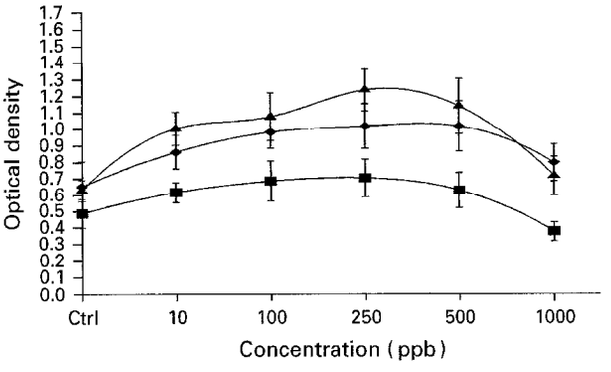

Figure 5 MTT assay with CRG/5 mol % Zn. The effect seen with the zinc-releasing glass was not cytotoxic; the exudate raised the metabolic rate of the cells up to 48 h.

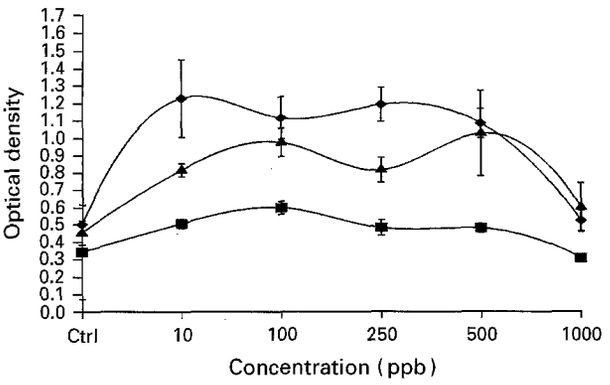

Figure 6 MTT assay with PVP. After 24 h the PVP has not detrimentally affected the viability of the cells.

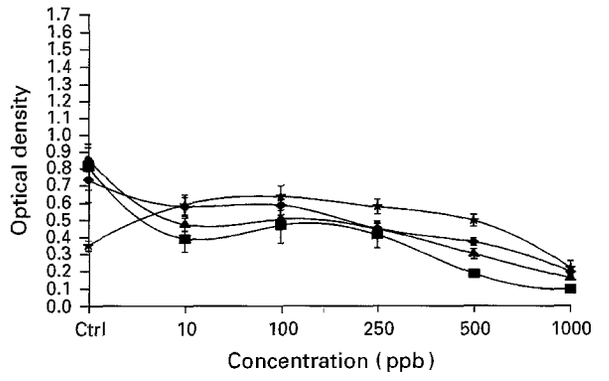

Figure 7 MTT assay with standard metal ion solutions after 72 h. The trend seen with the CRGs of a range of concentrations increasing cell growth can also be seen with the standard metal ion solutions.

there is a slight decrease in cell viability up to 250 p.p.b., when again the cell growth increases. This effect is seen until 1000 p.p.b., when the solution exhibits toxicity. This could be due to the components of the exudates having a critical range of concentrations where they stimulate cell activity.

The results obtained using the standard metal ion solutions after 72 h (Fig. 7) show that the solutions are more toxic overall than the exudates containing the CRGs. The impact of having the maximum level of metal ions available immediately the solutions make contact with the cells will affect their growth more detrimentally than a steady release of the same concentration of ions over a time period. As with the CRGs, where there was a range of concentrations which seemed to encourage cell growth, the same effect can be seen with the standard metal ion solutions.

4. Conclusions

It can be seen that the controlled metal ion releasing glasses sustain cell growth, if not increase the rate of cell division, whereas the antibacterial agent chlorhexidine produces irreversible cell damage at low concentrations.

As the glasses are releasing the metal ions over a period of time, the levels of the ions will initially be lower. This may explain why the copper- and silver-releasing glasses became toxic at concentrations much higher than expected and the marked contrast with the results obtained with the standard metal ion solutions.

When these results are applied to the functions of a wound dressing, the glasses have potentially far-reaching consequences. As the glasses can be manufactured in many different forms they can easily be incorporated into wound dressings. If bacteria were present at the wound site the benefits from utilizing the glasses would be that the released metal ions would inhibit the proliferation of bacteria whilst the division and growth of cells may be promoted by the presence of the metal ions. Therefore the glasses may also be beneficial for non-infected wounds. This would decrease the need for systemically administered antibiotics in the advent of infection and there would be no possibility of antibiotic-resistant strains of bacteria developing.

Acknowledgement

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