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A latex membrane, as an alternative device in the GTR technique: preliminary report on its biocompatibility

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Polymeric materials are of widespread use for many clinical applications, including dentistry. Periodontal guided tissue regeneration (GTR) is a technique developed to treat periodontal disease: it implies that new connective tissue attachment and bone regeneration are achieved, whereas epithelial migration is prevented by the placement of a barrier membrane. This study has focused on the toxicity *in vitro* of different polymeric membranes, either commercial or experimental, with a latex rubber membrane being of special interest for use as an implant material in guided tissue regeneration in periodontology. A cell culture system tested by quantitative assay methods offered a reliable tool which provided meaningful results on the first level biocompatibility of such membranes.

1. Introduction

The predictable regeneration of a new attachment apparatus, after the natural attachment has been destroyed by periodontal disease, is one of the most challenging problems in dentistry [1]. Recent studies [2, 3] have demonstrated that extensive regeneration of the attachment apparatus occurred with the use of a surgical procedure called guided tissue regeneration (GTR).

This procedure includes the placement of a membrane between the gingiva and the root surface: this device serves as a barrier preventing the gingival epithelium from having contact with the root during healing, and at the same time, giving preference to cells originating from the periodontal ligament and bone, to repopulate the wound area adjacent to a previously exposed root surface.

Several types of membranes (resorbable and not resorbable) were proposed and developed in order to achieve the periodontal regeneration. The first available device specially designed for GTR was made of expanded polytetrafluoroethylene (e-PTFE), and the most commonly used resorbable materials, as reported in the scientific GTR literature, have been collagen, polyglycolic acid, polylactic acid and copolymers of these materials [4].

Ideally, the barrier membranes should be biocompatible, establish a marginal seal to exclude or to retard epithelial cell migration and bacterial contamination, be manageable, but also rigid for the space maintenance, have different shapes according to the multiple periodontal defects and, finally, be quite cheap.

Currently, materials of potential use in dentistry are limited, being very expensive and unable to seal off the different shapes of the periodontal compromised roots, with concavities, furcations, fluting, etc., and multiple bony defects.

Recently some case reports [5, 6] have shown the possibility of utilizing latex barriers: their advantages include manageability, adaptability to the root surface, different design, water repellency and low cost.

Unfortunately, studies have not yet been carried out comparing the biocompatibility of latex membranes with that observed for other membranes clinically used.

The purpose of this study was to compare the biocompatibility of a new latex membrane with other membranes including GORETEX®, VICRYL* and PAROGUIDE®, widely used in clinical practice, using cell culture techniques.

To ensure the biological safety of materials, simple and reliable *in vitro* biocompatibility testing methods are needed: cell culture methods provide a useful tool to satisfy this need. The methods employed include: neutral red uptake assay, which measures cell viability, being absorbed only by viable cells; MTT test, which also detects cell viability by measuring the reduction of tetrazolium salts by mitochondrial enzymes of viable cells; total protein content and crystal violet staining of the cells, which both provide an indirect measurement of cell growth.

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2. Materials and methods

2.1. Test materials

The following materials were assayed:

- PAROGUIDE®, a resorbable collagen membrane, supplied by Coletica, Lyons (France).
- VICRYL* periodontal mesh, made of resorbable Polyglactin 910, supplied by Johnson & Johnson, Skillman, New Jersey (USA).
- GORETEX® periodontal material or augmentation material made of polytetrafluoroethylene, supplied by W.L. Gore & Associates, Inc., Flagstaff, Arizona USA.
- Synthetic polymeric elastomer (SPE): pieces were obtained by cutting surgical dental gloves (Regent Co., London, UK).
- Experimental natural latex membrane following extensive leaching (Sample 1) (Regent Co., London, UK).
- Experimental natural latex membrane with cetylpyridinium-chloride added as antibacterial agent (Sample 2): pieces were obtained by cutting surgical dental gloves (Regent Co., London, UK).
- Foley's cathether silicone elastomer, supplied by Amplimedical, Assago (Italy).
- Natural rubber latex from single use urethral catheters (NRL), supplied by Amplimedical, Assago (Italy).

The latter two materials were chosen as negative (i.e. non-toxic) and positive (i.e. toxic) control materials, respectively [7, 8].

2.2. Cell culture

L 929 cells (ATCC, NCTC clone L929), i.e. fibroblast-like cells from an established cell line derived from mouse areolar and adipose tissue, are extensively being used in biocompatibility testing [9]. This line was propagated in 75 cm^2 tissue culture flasks by culturing in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum, 2 mM glutamine, non-essential amino acids, 7.5% bicarbonate buffer, 100 units/ml of penicillin and 100 μg/ml streptomycin.

Following subculture, cells were plated in cell-culture treated polystyrene plates (time 0) 4×10^5/ml MEM in 24-well plates and 5×10^4/200 μl MEM in 96-well microtitre plates and allowed to grow. Pieces of sterile test materials were added after 24 h of culture as follows: 10 mm^2 and 4 mm^2 pieces to 24-well and 96-well cultures, respectively. All materials were tested in duplicate (24-well cultures) or triplicate (96-well cultures). After an additional 24 h culture period, test materials were removed and cells were processed according to the test method.

2.3. Test procedures

2.3.1. Neutral red assay

Neutral red was dissolved in Eagle's MEM 67 μg/ml (w/v), filtered through 0.45 μm filters and dispensed 100 μl/well into 96-well microcultures after removal of the supernatants. Following a 2 h incubation at 37 °C, the neutral-red-added medium was discarded and, after washing the cells twice with saline, the dye incorporated by the cells was extracted with 100 μl of 50% ethanol in 1% acetic acid. Upon shaking of the plates, colour development in the microtrays was quantified at 540 nm wavelength.

2.3.2. MTT test

The procedure was recently described in [10]. Briefly, to each 96-well microculture was added 20 μl of MTT solution (1 g/5 ml phosphate-buffered saline), which was then incubated for 3 h at 37 °C. After discarding the supernatants, the reduced formazan inside the cells was solubilized with 100 μl dimethylsulphoxide and the absorbance read at 540 nm.

2.3.3. Total protein content assay

The assay was based on the Lowry method, after modification to adapt to 24-well microcultures. Spectrophotometric readings of the developed colour were made at 750 nm.

2.3.4. Crystal violet assay

The 24-well cell monolayers medium was replaced by 0.5 ml of crystal violet 0.25% in methanol for 10 min. Upon discarding the solution, the stained cells were treated with lysing solution (0.95 ml HCl 1N and 0.8979 g sodium citrate in 98.05 ml ethanol 47.5%) for 30 min and the absorbance read at 490 nm.

2.3.5. Data presentation

Results were recorded as optical density: after subtraction of the blank, the arithmetic mean of the replicate samples was calculated. Data are presented as the proportion of viability/growth of each material-treated culture compared to its control culture, arbitrarily set to 100. Expressing the data in such as way allows for comparison between different experiments.

3. Results

Cell monolayers representative of the cultures with neutral red are shown in Figs 1–3. No qualitative differences were observed between the 24 h cultures for silicone or GORETEX® (Figs 1 and 2, respectively). In contrast, the cells exposed to Sample 2 were very unhealthy (Fig. 3).

Cell viability following 24 h contact with the materials tested was measured by the neutral red uptake method in five separate experiments: triplicate samples for each material were assayed.

The results obtained allowed the ranking of materials in order of relative toxicity: VICRYL* and PAROGUIDE® membranes are the least toxic of the eight polymers tested, as they behave no differently from negative reference material, i.e. silicone. GORETEX® and Sample 1 apparently have almost the same activity, while SPE slightly affects cell viability. Sample 2 was undoubtedly the most cytotoxic, often approximating the values recorded for positive control material, i.e. natural rubber latex (Table I).

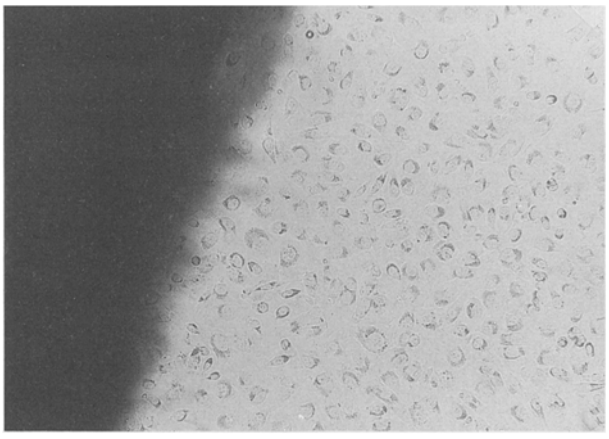

Figure 1 Light microscopy of L929 cells close to a silicone sample in a 24 h-culture stained with neutral red. Cells appear to be numerous, elongated and healthy (neutral red, original magnification × 10).

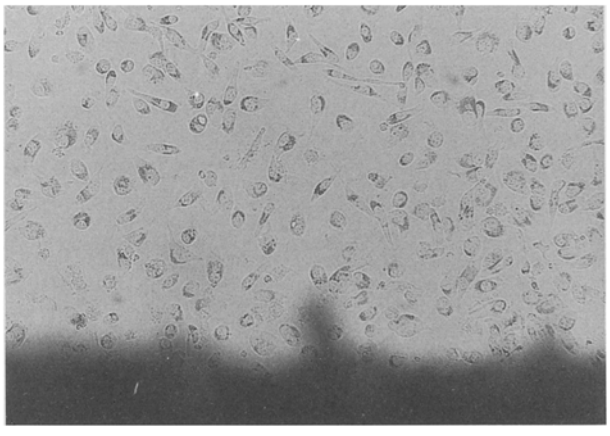

Figure 2 Light microscopy of L929 cells close to a GORETEX® sample in a 24 h-culture stained with neutral red. Cells show the same appearance as Fig. 1 (neutral red, original magnification × 10).

Figure 3 Light microscopy of L929 cells close to Sample 2 in a 24 h-culture stained with neutral red. Rare cells are rounded, scarcely stained and unhealthy (neutral red, original magnification × 10).

The MTT test, which also measures cell viability, was used for a single experiment (with triplicate samples): the results agreed perfectly with previous data (Table II).

TABLE I Neutral red uptake assay performed on L929 cells after 24 h exposure to different membrane samples. Results of five separate experiments with triplicate samples are expressed as percentage changes versus control (= 100%) (n.p. = not performed).

Control	100	100	100	100	100
Silicone	100	100	81	106	114
NRL	50	26	34	42	35
Paroguide®	96	96	86	106	133
Vicryl®	n.p.	96	n.p.	106	122
Goretex®	83	100	78	108	75
SPE	83	79	58	86	55
Sample 1	91	74	81	95	112
Sample 2	47	6	< 1	< 1	< 1

The results from the assay of total protein content is reported in Table III: duplicate samples were assayed in three separate experiments. The same trend was found: all the commercial materials, SPE and, obviously, the negative control (i.e. silicone) scored values ranging from 70 to 95. Sample 1 allowed slightly less cell growth; NRL and Sample 2 undoubtedly had the most striking cytostatic effect.

By using the crystal violet assay, cell growth under the influence of the test materials was assessed and the results from three independent experiments with duplicate samples are shown in Table IV. High values were recorded—which means cell growth is unaffected—for all materials except Sample 2, which scored values ranging from 18 to 49, and, not unexpectedly, NRL.

4. Discussion

The biocompatibility of six different membranes, fabricated from both synthetic and natural materials, was assayed by cell culture systems in order to verify the potential acute toxicity of such devices. The aim of this work was to obtain indications for selecting a latex-rubber-based material which could be used for GTR.

The membranes tested produced different cytotoxic effects, possibly linked to their chemical composition as well as to the addition of different substances, including vulcanizing accelerators, antioxidants, etc. It has been shown that these substances, when added to natural latex rubber materials, often cause the materials to exhibit strong cytotoxicity [11, 12]; therefore these materials are recommended as positive controls for cytotoxicity testing [13].

Unfortunately, detailed information on substances added to the materials tested are extremely difficult to obtain.

Qualitative assessment of the cell cultures was performed by light microscopy: commercially available membranes, SPE elastomer and Sample 1, as well as silicone, exhibited negligible cell death at 24 h. At the same endpoint, the cells were lysed in the immediate vicinity of Sample 2 material.

The quantitative results correlated well with the qualitative observations.

It is to be noticed that the four different quantitative test methods were all able to detect differing levels of

TABLE II MTT test performed on L929 cells after 24 h exposure to different membrane samples. Results of a single experiment with triplicate samples are expressed as percentage change versus control (= 100%).

Control	Silicone	NRL	Paroguide®	Vicryl®	Goretex®	SPE	Sample I	Sample 2
100	70	43	84	92	110	73	82	1

TABLE III Total protein content assay performed on L929 cells after 24 h exposure to different membrane samples. Results of three separate experiments with triplicate samples are expressed as percentage change versus control (= 100%) (n.p. = not performed).

Control	100	100	100
Silicone	70	91	92
NRL	42	51	n.p.
Paroguide®	73	92	95
Vicryl®	n.p.	n.p.	n.p.
Goretex®	76	91	80
SPE	73	93	92
Sample 1	79	80	78
Sample 2	55	50	45

TABLE IV Crystal violet assay performed on L929 cells after 24 h exposure to different membrane samples. Results of five separate experiments with triplicate samples are expressed as percentage change versus control (= 100%) (n.p. = not performed).

Control	100	100	100
Silicone	97	102	103
NRL	40	35	32
Paroguide®	91	101	113
Vicryl®	94	108	92
Goretex®	90	109	95
SPE	98	106	n.p.
Sample 1	92	110	106
Sample 2	27	18	49

toxicity, which means they are reliable procedures for testing cytotoxicity.

The total protein content assay results were the least sensitive, with all materials having similar values, slightly decreased compared to the other assays. It may be hypothesized that proteins or phenols leached by rubber samples could have been interfering with the Lowry assay. Nevertheless, the methods were generally in good agreement and the ranking of the materials tested for toxicity was the same with each method.

All the commercial materials, including the SPE elastomer whose composition was unknown, have proved to be cell-compatible. It should perhaps be noticed that the resorbable materials, that is collagen-based PAROGUIDE® and polylactic acid-based VICRYL®, showed the greatest compatibility.

Following a cumulative analysis of the data obtained in this study, it could be concluded that between the rubber-latex-based materials whose cytotoxicity was unknown, that is Sample 1 and Sample 2, the first one is more biologically compatible. Our results clearly indicate the existence of marked variations in cytotoxicity between various latex products. The toxicity of our sample is probably due to the antibacterial agent added to the gloves (Sample 2), while the extensive leaching performed on Sample 1 has been effective in removing extractable toxicants. The choice of Sample 1 for clinical application is justified by the fact that overt toxicity is not necessary for the function required. The employment of materials exerting a severe toxic effect on cells would not only discourage the *in vivo* colonization of bone by an epithelial cell lining (which would mean that the material has accomplished the function it is intended for), but could also stop the attachment and proliferation on to the bone of viable fibroblasts to achieve a new attachment apparatus. The latter activity would hamper stable fixation of the bone, with an increased risk of implant failure.

In conclusion, the biocompatibility of rubber latex membranes is highly variable due to large difference in the additives used in the manufacturing of such materials: our cell culture tests have successfully screened synthetic and natural materials for potential toxicity *in vivo*. Although the *in vitro* results are not able completely to predict the *in vivo* performance of materials, these promising results indicate a strong potential for latex rubber material biocompatibility, and *in vivo* studies are warranted to establish its usefulness for GTR.

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