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In Vitro Toxicity Test of Ethyl 2-Cyanoacrylate, a Tissue Adhesive Used in Cardiovascular Surgery, by Fibroblast Cell Culture Method

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ABSTRACT

Background: The aim of this study was to evaluate the cytotoxicity of cyanoacrylate polymers (ethyl 2-cyanoacrylate) by an elution test system. In such systems, the material is extracted with a cell culture medium, which is subsequently added onto cultured cells, resulting in an indirect contact between the biomaterial and cells.

Methods: A cell line commonly utilized for cytotoxicity experiments; L929 mouse fibroblasts were used in this study. The effects of extract dilutions on cells were evaluated by two experiments: (a) The cells were suspended and seeded in a medium containing the extract, followed by a short incubation to observe the effects on cell attachment; (b) cells were seeded in a normal medium. Following cell attachment, this was replaced with a medium containing the extract and long-term effects on cell proliferation were measured. The cytotoxicity was quantified using a cell viability assay, well established for use in the evaluation of cell–biomaterial interactions.

Results: These results indicate that, in the test system utilized, a tenfold dilution of the extract results in an ~10% decrease in cells; this increases to between 30% and 45% in a 1:1 dilution. When a large number of cells (3000/well) were used, proliferation of cells overcame the cytotoxic effect and consistent results could not be observed.

Conclusions: In this study, the observed outcomes follow a similar trend on cell attachment and proliferation with acute effects (4 hours incubation) of the extracts on the cells, producing slightly higher toxicity. Our findings are parallel with the literature findings.

INTRODUCTION

Cyanoacrylate (CA) polymers are adhesives with unique features, such as the ability to bond in an aqueous environment [Brauer 1979] and biodegradability. These have been utilized as tissue adhesives [Weber 1984] and for the aim of hemostasis [Leonard 1967]. For cardiovascular surgery, uncontrollable hemorrhage, impaired tissue integrity, sternal dehiscence, and for pulmonary surgery, continuing air leakage are significant causes of morbidity and mortality. Occasionally, solely conventional techniques such as classical suturing and patch application are not sufficient to stop bleeding and tissue adhesives, which may be effective across large surfaces, may be needed [Eastman 1998, Horsley 1997, Werker 1997]. In our clinic, we use ethyl 2-cyanoacrylate as a tissue adhesive [Demirtas 1998, 1999] for repairing the tissues, together with a pericardial patch, an expanded polytetrafluoroethylene patch (ePTFE, Impra, Inc, Tempe, AZ, USA), or Teflon felt, and we obtain good results.

Although numerous reports document the in vitro and in vivo toxicity of these materials [Ciapetti 1994, Evans 1999, Thumwaint 1999], recent studies indicate the successful utilization of CAs in surgical procedures and acute hemorrhagic conditions [Applebaum 1993, De Blanco 1994, Leahey 1993]. The possibility of cytotoxicity requires the in vitro evaluation of these compounds.

The aim of this study was to evaluate the cytotoxicity of CA polymers by an elution test system. In such systems, the material is extracted with a cell culture medium, which is subsequently added onto cultured cells, resulting in an indirect contact between the biomaterial and cells [Werker 1997].

MATERIALS AND METHODS

A cell line commonly utilized for cytotoxicity experiments [Cenni 1999]: L929 mouse fibroblasts were used in this study. The effects of extract dilutions on cells were evaluated by two experiments: (a) the cells were suspended and seeded in a medium containing extract, followed by a short incubation to observe effects on cell attachment; (b) cells were seeded in normal medium. Following cell attachment, this was replaced with a medium containing extract and long-term effects on cell proliferation were measured. The cytotoxicity was quantified using a cell viability assay well established for use in the evaluation of cell–biomaterial interactions [Nishi 1995].

Cell Culture

L929 mouse fibroblasts were grown in DMEM/F12 medium containing 10% fetal calf serum, 100 IU/mL peni-

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cillin and 0.1 mg/mL streptomycin (Biological Industries, Israel) buffered with $NaHCO_3$ (complete DMEM/F12 medium) at 37°C in a 5% CO_2-95% air atmosphere incubator. Cells were passaged at a 1:6 ratio upon reaching confluence. Experiments were performed with cells from passages 10-15.

Materials

A CA of unknown composition in fluid form, sold commercially, was used in the tests. For the elution test, the CA was extracted in a complete DMEM/F12 medium (15 µL:5 mL) at 37°C for 120 hours in a glass tube [Werker 1997], in line with ASTM practice F 619-79.

For the cell attachment assay, the extracts were diluted at varying ratios (v/v; 9.5:0.5, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 2:8) with fresh complete DMEM/F12 medium, and cells were seeded at 5 or 10 $\times 10^3$/well/0.2 mL in these dilutions into flat-bottomed 96-well micro plates (Techno Plastic Products, Switzerland) and allowed to adhere for 4 hours.

For the cell proliferation assay, cells were seeded at 1, 2, or 3×10^3/well/0.2 mL in flat-bottomed 96-well micro plates and allowed to adhere for 18 hours; thereafter, the medium was removed and extracts diluted with fresh complete DMEM/F12 medium at varying ratios (v/v; 9.5:0.5, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 2:8) were added, followed by incubation in a CO_2 incubator for another 48 hours. For the attachment and proliferation assays, five replicates were seeded for each extract dilution point into micro plates. Cells placed in complete DMEM/F12 medium served as controls.

Cell Growth Experiments

The amount of cells present in the wells following incubation with the extracts was quantified using a neutral red uptake assay [Guo 1998]. Cells present in the wells were washed once with sterile PBS and incubated with PBS containing 2% FCS and 0.001% neutral red for 2 hours in the incubator, enabling the uptake of the dye into viable cells by an active process. Following this incubation, a brief wash with 4% formaldehyde in PBS, resulted in the fixation of the cells. The dye retained by the cells was solubilized in 2 mL of 50% EtOH, 1% acetic acid in H_2O. The level of the dye was measured spectrophotometrically at 540 nm, the absorbance was found to be proportional to the number of viable cells in each well.

RESULTS

The effect of the various extract dilutions on the L929 attachment was investigated by seeding 5 or 10×10^3 cells/well. As seen in Figure 1, a 1.5:8.5 dilution of the extract in complete DMEM/F12 medium resulted in a 12% decrease in the cell attachment relative to the control, whereas a 40% decrease was observed in 1:1 dilution. For 5000 cells/well, a 12% decrease in the cell attachment relative to the control 6:4 dilution of the extract in complete DMEM/F12 medium was observed, and the decrease was found to increase to 45% in a 1:1 dilution.

To investigate their effects on cell proliferation, the extract dilutions were added onto attached L929 cells and proliferation following 48 hours incubation was compared to controls

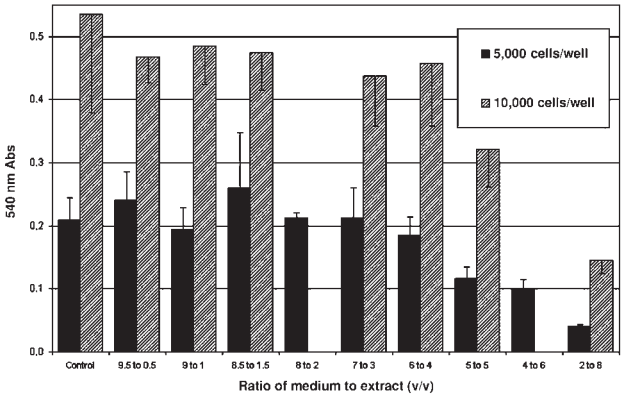

Figure 1. L929 Cell Attachment by Neutral Red Assay 4 h incubation with extract: Effect on cell attachment: For the cell attachment assay, the extracts were diluted at varying ratios (v/v; 9.5:0.5, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 2:8) with fresh complete DMEM/F12 medium and cells were seeded at 5 (■) or 10 (▨) $\times 10^3$/well in these dilutions into 96-well micro plates and allowed to adhere for 4 h, followed by the neutral red assay.

(Figure 2). In experiments with 1000 and 2000 cells/well, 1:9 dilution of the extract in complete DMEM/F12 medium resulted in 12 and 18% decreases in cell attachment relative to the controls, whereas 38% and 30% decreases were observed in 1:1 dilution, respectively.

These results indicate that, in the test system utilized, a tenfold dilution of the extract results in an ~10% decrease in cells, and this increases to between 30% and 45% in a 1:1 dilution. When large number of cells (3000/well) were used, proliferation of cells overcame the cytotoxic effect and consistent results could not be observed.

Discussion

There are studies on the use of tissue adhesives in aortic surgery, particularly for controlling bleeding from the suture lines between native aortic tissue and synthetic grafts, for joining rough and fragmented surfaces, for easing the anastomosis of tubular structures, and for supporting unreliable suture lines. CA derivatives are among these tissue adhesives, which may be used in cardiovascular and pulmonary surgery when necessary. According to the comparative trials with biological adhesives, CA may be complementary for or an alternative to the classical suture technique due to its strong bonding upon application, despite its low elasticity [Werker 1997, Horsley 1997].

The various instances of our operations, in which we used ethyl 2-cyanoacrylate as a tissue adhesive for repairing the tissues, were as follows: in thoracic aortic surgery, when there is need to support anastomotic line or aortotomy (22 patients), in redo sternotomy when laceration of right ventricular free wall occurs during dissection (9), after resection of left ventricular aneurysm (4), bleeding from proximal anastomosis during coronary bypass surgery (4), bleeding following left ventriculotomy performed for hydatid cyst (1), in superior vena cava laceration (1) and in rupture of coronary sinus (1), in sternal dehiscence (16), in continuing pulmonary air leakage (5), and bleeding from femoral artery due to femoral epidermoid carci-

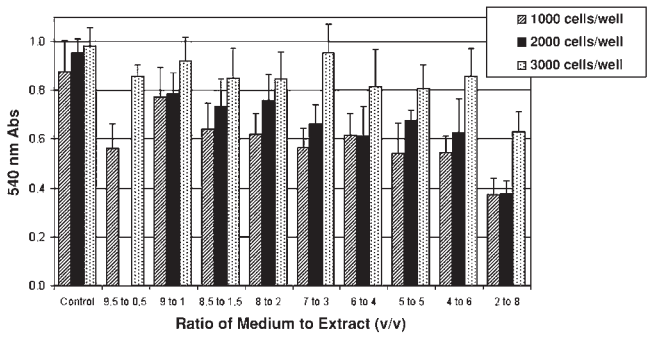

Figure 2. L929 Cell Proliferation by Neutral Red Assay 48 h incubation with extract: Effect on proliferation: For the cell proliferation assay, cells were seeded at 1 (⁝⁝), 2 (■), or 3 (▨), $\times 10^3$/well into 96-well micro plates and allowed to adhere for 18 h, thereafter the medium was removed and extracts diluted with fresh complete DMEM/F12 medium at varying ratios (v/v; 9.5:0.5, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 2:8) were added, followed by incubation in a CO_2 incubator for another 48 h.

noma (1 patient). We have used it in a total of 64 patients (23 female; mean age 53.6 ± 9.7 years). Ethyl 2-cyanoacrylate application was successful in 61 patients (95.3%).

This in vitro study is the last one of the studies, which we realized, was relevant to ethyl 2-cyanoacrylate. In one of the before studies, we investigated experimentally the possible histopathological effects of ethyl 2-cyanoacrylate glue when used as a tissue adhesive in cardiovascular and thoracic surgery. As no significant difference between conventional suture and ethyl-2-cyanoacrylate application was detected in terms of histopathological reactions, ethyl 2-cyanoacrylate may be considered as an alternative or adjunct to conventional techniques in controlling hemorrhage that cannot be done by conventional methods, as well as in tissue repair and in the control of pulmonary air leakage, and in vascular (ascending and abdominal aortic), myocardial and pulmonary surgery [Kaplan 2004a].

In another study that we realized, we investigated absorption of ethyl 2-cyanoacrylate glue when used as a tissue adhesive. Ethyl 2-cyanoacrylate was applied subcutaneously to four rats; its presence in blood and urine was investigated by using High Pressure Liquid Chromatography. Blood samples were drawn at baseline and after 2, 4, 6, 24, 48, 54, 78, and 96 hours following application. Urine samples were obtained at baseline and after 4, 24, 48, 72, and 96 hours. Administration of ethyl 2-cyanoacrylate resulted in its absorption of unchanged ethyl 2-cyanoacrylate and unknown metabolites, in plasma and urine [Kaplan 2004b].

In another study that we realized, we investigated microbial contamination risk and antibacterial effect of ethyl 2-cyanoacrylate. Ethyl 2-cyanoacrylate was not found to be contaminated during repeated uses or following direct bacterial inoculation. With this study, it was concluded that ethyl 2-cyanoacrylate has an antibacterial effect against bacteria, frequently isolated in surgical site infections [Yavuz SS].

CONCLUSION

In this cell culture study, the results of this study indicate that a tenfold dilution of the extract results in an ~10% decrease in cells. When a large number of cells was used, proliferation of cells overcame the cytotoxic effect and consistent results could not be observed.

In this study, the observed outcomes follow a similar trend on cell attachment and proliferation with acute effects (4 hours incubation) of the extracts on the cells producing slightly higher toxicity. Testing two CA surgical glues and utilizing the neutral red assay, Montanaro and colleagues [2001] have reported that these were nontoxic to L929 cells only when diluted to 1:10 with culture medium, a result in parallel with our findings.

In conclusion, in the light of previous experimental and in vitro studies and according to our in vitro cell culture study, we concluded that the results of these studies support the clinical usage of ethyl 2-cyanoacrylate as a tissue adhesive in cardiovascular and thoracic surgery.

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