Introduction

*Acrylic denture base is a major form of prosthesis for people living in the developing world since it is inexpensive, but acrylate can be problematic for patients when it becomes broken and they repair the denture rather than having a new one made. Some dentists have had the experience of patients repairing their own dentures using commercial cyanoacrylate (CA) adhesive or 'super glue'. CA adhesive is a compound synthesized by condensation of a cyanoacetate with formaldehyde in the presence of a catalyst. Nowadays, this CA has also been used successfully as a tissue adhesive in many types of surgical and wound repair[1-3] and for the control of hemorrhage[4-5]. There have been some studies which have showed that CA when used as tissue adhesive can cause inflammation and tissue necrosis in vivo[6-7] and that it is cytotoxic to cells in vitro. These studies used cell culture with direct contact of this compound[8] or an extract of the CA[9].

In this study, the cytotoxicity of substances that may be released from polymerized commercial CA adhesive available in the marketplace, which patients may buy to repair their denture, were investigated by using human oral fibroblast cells from primary culture and exposing them to the polymerized adhesive in filter paper. The pattern of toxicity of this polymerized CAs in filter paper in the culture media when incubated for various period of time was also investigated.

MATERIALS AND METHODS

Cell Culture

Human oral fibroblast-like cells were obtained from the gingival tissue of the patients who had surgical removal of impacted teeth. After washing the tissue with an iodine-based antibacterial agent, the mucosal layer was stripped to remove keratinocyes using a scalpel blade and scissors. The tissue was then cut into small pieces following the primary explant technique[10] which utilized Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL, New York, USA) with 10% fetal calf serum (Seromed , Berlin , Germany), 100 units/ml Penicillin, 100 μg/ml Streptomycin (Gibco BRL, New York, USA), and 1% amphotericin B. The resulting material was cultured at 37 °C with 5% CO_2 , 95% air. After the outgrowth of cells from the explants covered nearly all the culture plate, subculture was done by 0.25% Trypsin (Gibco BRL, New York, USA). Cells used for the cytotoxicity test were taken from the subculture between the fourth to twentieth passages .

Materials

2-Cyanoacrylic acid esters (Sigma, MO, USA), which is approximately a 1:1 mixture of ethyl and methyl esters, were used as the standard cyanoacrylate (std CA).

Three different brands of commercial cyanoacrylate adhesives in liquid form were purchased from local markets in Thailand and were coded as:

CA1 - Yamayo (Super Glue), Japan

CA2 - Alteco (Super Glue) made from Alpha Techno group, Japan and Singapore

CA3 - KenJI brand made from Taoka chemical company, LTD, Japan, which is stated to be composed of α-Cyanoacrylate.

Number 1 Whatman filter papers (Sringfield, Kent, England) were cut in a half circular shape with a diameter of 6 mm (this diameter was smaller than the diameter of the well in 96 multiwell culture plate so that the media will be exposed to CO_2 and air). The paper was autoclaved, then saturated with cyanoacrylate adhesive by adding about 5 µl of the adhesive. It was then left for 24 hours to allow full polymerization. Fully polymerized cyanoacrylate will float in the media, hence two control systems were used, one with cells and normal media and the other with paper coated with wax to imitate the cells in the test material so that the paper floats over them.

Methods

The cytotoxicity of substances that may be released from polymerized commercial cyanoacrylate adhesive was compared to standard cyanoacrylate by observation of cell

morhology, which included the zone of inhibition, and cytotoxicity was measured by MTT method. Substances released from CA-coated paper were stained with crystal violet and allowed to stand for 24 hours and then compared to controls, which included the wax-coated paper. Crystal violet staining was also used to investigate cytotoxicity of releasing substance from CA-coated paper that was incubated in the culture media at 37 °C with 5% CO_2 , 95% air for 1 day, 3 days, one week and two weeks, with the media changed daily.

Cell morphology and migration

Oral fibroblast cells were subcultured and approximately 1×10^5 cells were plated out on 35 mm culture dishes that already had one circular shape with diameter 6 mm. filter paper saturated with cyanoacrylate attached at the middle of the dish. These were left for 24 hours to allow full polymerization. The cells were also plated to the culture dish which had wax coating applied to the middle of the dish as one control.

Also the same quanity of cells was added to the culture plate without any test material applied to act as a further control. Culture medium was changed every 2 days and the morphology of cells were observed with an inverted microscope.

Cytotoxicity

MTT test

This method was first described by Mosmann in 1983[11]. Cells were subcultured and seeded to 96-well microtitre plates at about 3500 cells/200 µl of media in each well, except the blanks. After 24 hours, filter paper with each type of CA and wax coating were placed (floated) into each well

except for the controls. After 3 days, the filter paper and media were removed and 200 µl of fresh media was added to each well including the blanks. Then 50 µl of 5 mg/ml of MTT (Sigma, M-5655) in sterile Phosphate buffer system (PBS) was added. The plate were then wrapped in aluminium foil and incubated at 37.5 C for 4 hours. The media was then removed and MTT and 200µl of DMSO were added in each well, followed with 25 µl of Sorensen's buffer solution. After mixing, the optical density was read against a blank at 590 nm using a microplate spectrophotometer (Titertek, Multiscan Plus MKII, Switzerland).

Crystal violet staining

This technique followed the method modified by Ikarashi, et al. (1993)[12]. After seeding the cells at the rate of about 3500 cells/200µl per well and leaving for 24 hours, the CA-coated and wax-coated filter papers were added in each well, except for the controls. After incubating for 3 days, the cells were fixed with 25% gluteraldehyde applied for 20 min. The cells were stained with 0.4% crystal violet in methanol for 30 min, rinsed with running water and then air-dried. Absorbances were read at 590 nm using the same microplate reader. The absorbance of the control wells which contained no test material was regarded as 100% and the percentage absorbance for each well was calculated.

This method also used to investigate cytotoxicity of substances that may be released from CA-coated paper after incubation with the media at 1 day, 3 days, one week and two weeks respectively.

Statistical Analysis

One way analysis of variance and Bonferroni multiple comparison were used to test the differences in optical density as the result of MTT and crystal violet staining methods for each of the tested materials. These tests were also employed for the analysis of the cytotoxicity test of each type of CA after incubation in culture media for various periods of time.

RESULTS

Cell Morphology and Inhibition Zone

After seeding cells in six 34 mm culture dishes, each type of polymerized CA-coated filter paper was attached to the middle of each of four of these culture dishes and one had the wax-coated filter paper attached. The remaining culture plate had only the cells set as the control.

After incubation for 24 hours, there was evidence of ruptured cells around all types of CA-coated paper, producing a zone of inhibition around each of the CA-coated filter paper, while the cells in the dish that had wax-coated paper maintained their fibroblast-like spindle shape and had begun to migrate and contact the wax-coated paper. The zones of inhibition around each of the CA-coated filter papers rangied from 200 μm to 1000μm. These inhibition zones persisted around each of the CA-coated filter papers for two weeks, even though media continued to replaced every 2 days (see Figures 1 to 3).

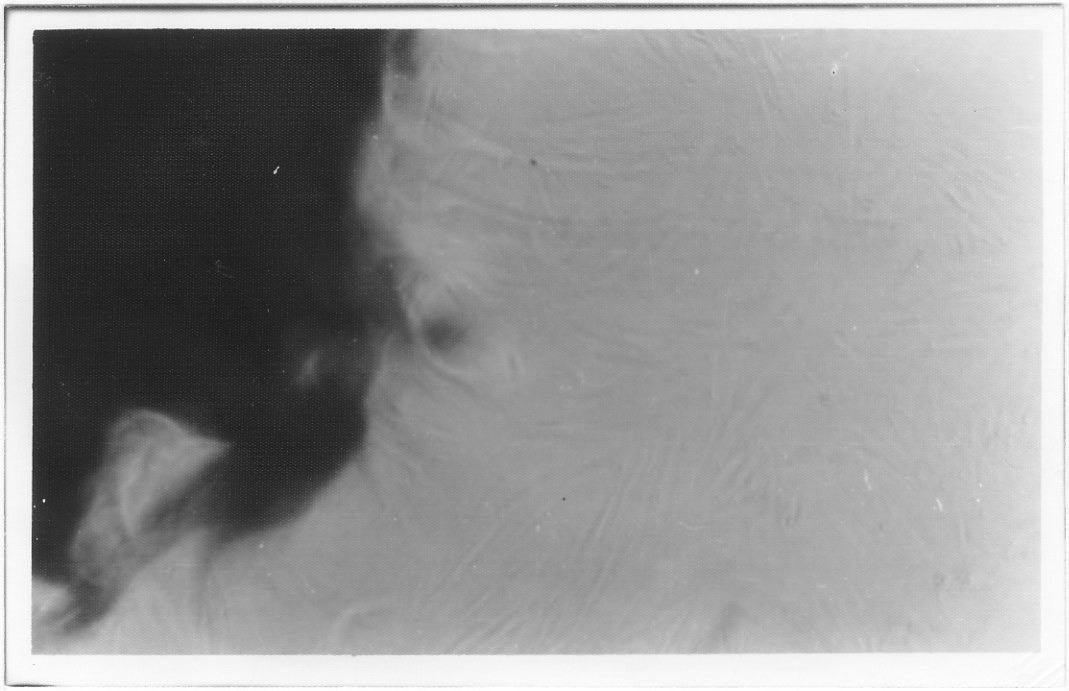

Figure 1. Oral fibroblast cells came close to wax coated paper after culture for 24 hours. (inverted microscope, original magnification × 10)

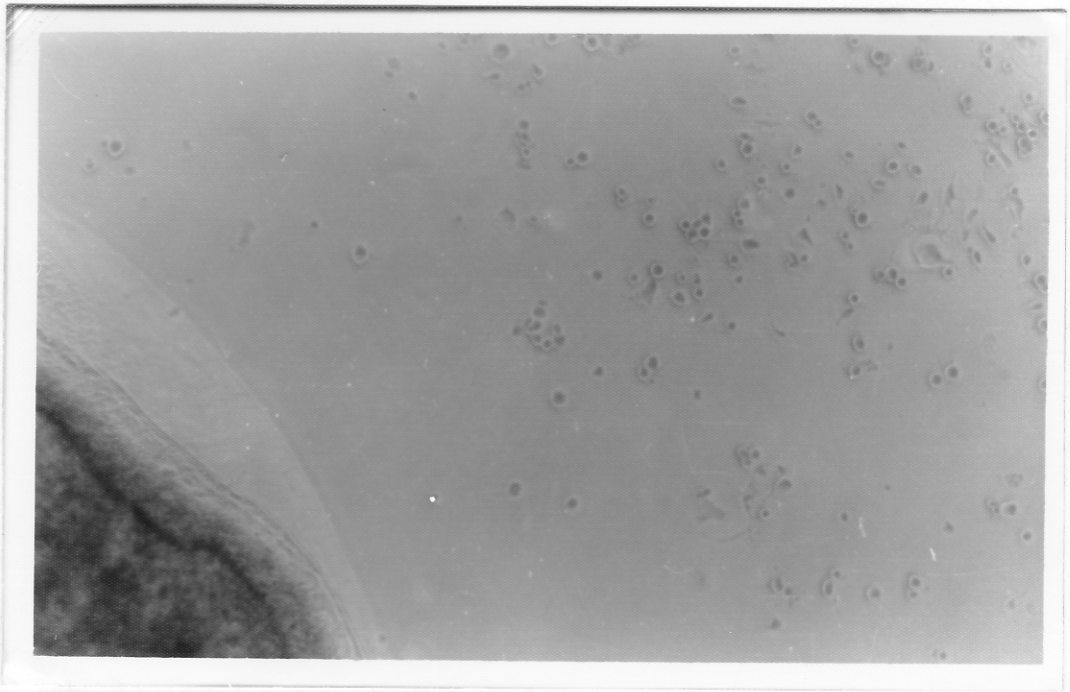

Figure 2. Cell apperance proximal to CA coated paper after 24 hours.

(inverted microscope original magnification × 4)

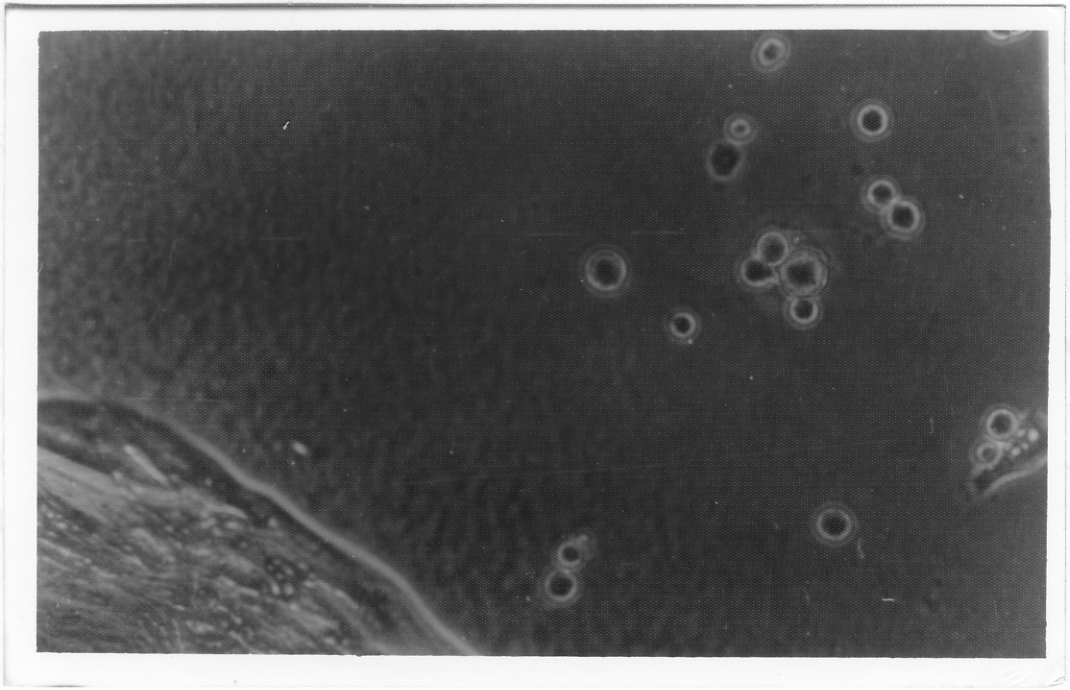

Figure 3. Cell appearance proximal to CA coated paper after 24 hours with higher magnification (inverted microscope original magnification × 10)

Cytotoxicity test

1. The polymerized CA adhesive compared with wax and control

MTT test

In the MTT test, the optical density (OD) of the cells treated with the three commercial CAs as well as the standard CA were significantly lower than the control and wax-coated filter paper ($p < 0.05$), but their OD were not significantly different from each other, when using the Bonforonni multiple comparison test. Wax-coated filter paper gave optical density nearly the same as the control (no significant difference).

(Table 1)

Crystal Violet Staining Method

The results from crystal violet staining method were similar to the MTT test, in that the cells which have commercial CA adhesive, including the std CA, gave absorbances of about half of the control, while the cells with the wax-coated filter paper gave an absorbance of about 96% of the control. Using the Bonforonni multiple comparison test, there was no significant difference between the percentage absorbance of all four CA, but they were significantly different from the wax-coated filter paper.

2. The releasing substances of CA after incubation in media for varying periods of time

There was no significant differences ($p > 0.05$) in the percentage absorbance comparing between the various time periods that the CA was allowed to float in the media in CA1, CA2 and CA3, except in the std CA where day 14 gave a significantly ($p < 0.05$) higher percentage absorbance

compared to day 1, 3 and 7. From Figure 3, the percentages of absorbance tended to be increased for std CA, CA2 and CA3 when compared to the cytotoxicity test of polymerized CA that were set for 24 hours and then tested without preincubation (Table 2) with the result of cytotoxicity test after incubation Cas for 1 day (Table 3), the optical density as perentage of control were increase about 73%, 37%,80% and 32% in CA1, CA2, CA3 and Std CA respectively.

Table 1. Cytotoxicity of polymerized cyanoacrylate adhesive compared with wax-coated filter paper and control using MTT test (n=10 in each group)

Test Material	Absorbance (against blank)
CA1	0.06±0.017
CA2	0.08±0.017
CA3	0.06±0.022
CA4	0.08±0.018
Std CA	0.09±0.015
Wax	0.43±0.058
Control	0.43±0.036
Wax	0.43±0.058
Control	0.43±0.036

Table 2. Cytotoxicity of polymerized cyanoacrylate adhesive coated filter paper compared with wax-coated filter paper using crystal violet staining method (n=30 in each group)

Test Material	Absorbance (% of control)
CA1	49.0±8
CA2	50.6±10
CA3	44.7±9
Std CA	46.8±7
Wax	96.0±13

Table 3. Mean ± SD (with 10 replications) of absorbance as a percentage of controls of incubated polymerized CA-coated filter paper in oral human fibroblast culture at day 1, 3, 7 and 14

Material	Time of incubation				P-value of
	1 day	3 days	7 days	14 days	ANOVA
CA1	84.8±23	76.8±25	81.7±13	76.0±12	0.7077
CA2	69.4±21	69.1±16	71.5±16	75.3±10	0.8190
CA3	80.8±23	95.5±18	81.7±20	87.3±13	0.2937
Std. CA	61.7±21	49.9±9	54.9±11	70.8±10	0.0104*

* Significant at $P<0.05$

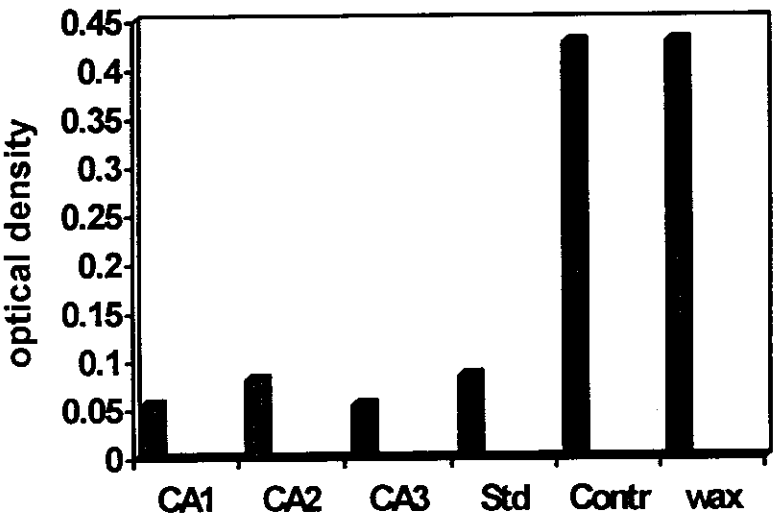

Figure4. MTT test on oral fibroblasts exposed to floating filter paper coated with various materials

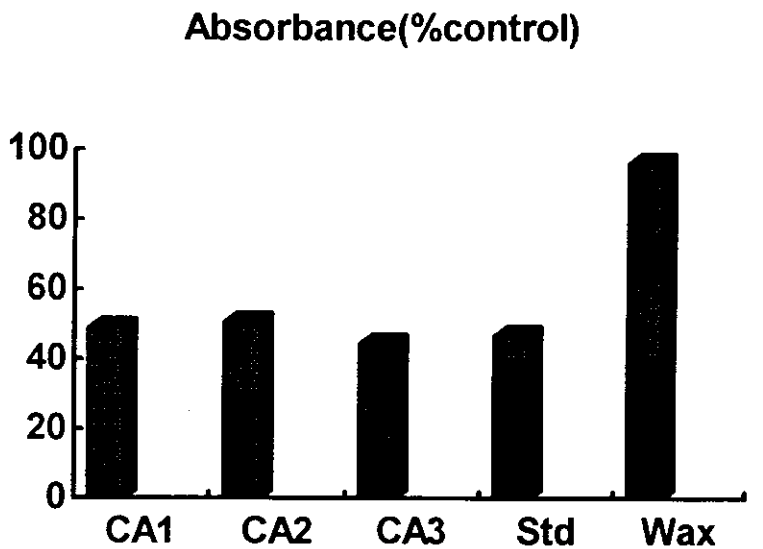

Figure 5. Absorbance of crystal violet staining of oral fibroblasts as percentage of controls

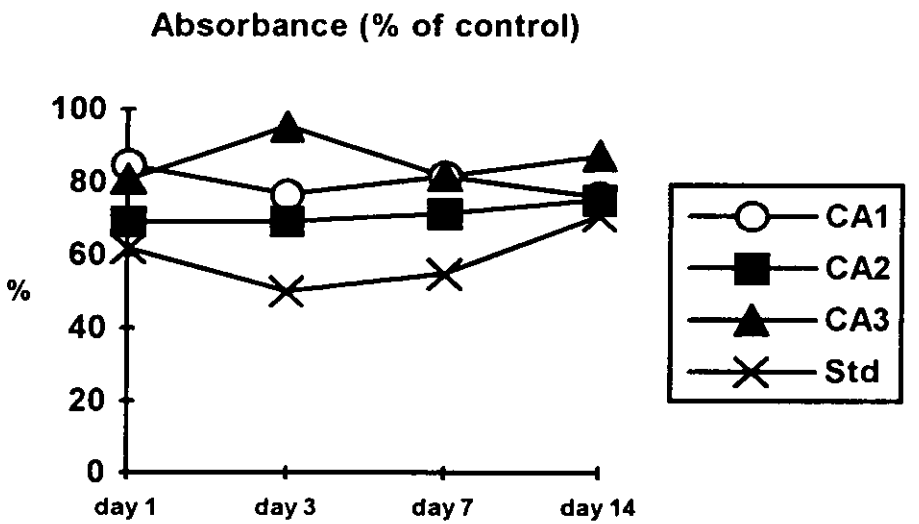

Figure 6. Absorbance of human oral fibroblast cells as a percentage of controls exposured to various types of cyanoacrylate-coated filter papers after incubation at day 1, 3, 7 and 14

DISCUSSION AND CONCLUSION

In this study, the cytotoxicity of substances released from polymerized commercial CA was investigated since this adhesive has been used by patients to repair broken acrylic dentures. Of concern was that toxic substances may be released that may be harmful to the mucosal tissue.

CA has been used as a tissue adhesive in surgery, for repairing wounds and to stop bleeding, but problems have been associated with CA derivatives due to the histotoxic effect on tissue. CA polymer has been shown to degrade to formaldehyde and cyanoacetate compounds which can accumulate in the treated area[13]. There is some evidence which shows that the formaldehyde released from CA is toxic to cells both in vitro and in vivo[14]. Also, with the higher release of isobutanol than formaldehyde as a consequence of in vitro degradation,of isobutyl CA[15], the degradation rate was decreased with increasing alkyl chain length from methyl-,ethyl-,isobutyl-, to isohexylcyanoacrylate particles[16]. Hence, there is a slower degradation process releasing fewer toxic byproducts per unit of time with the longer alkyl chain molecules compared with the faster rate with the shorter alkyl chain molecules.

In this study, filter paper was used as the vehicle for the adhesive to avoid direct cell contact with the adhesive, since, when CA sets, it produces heat which may be harmful to the cell culture. Testing the cytotoxicity of wax-coated filter paper was done to confirm that paper when floated above the cells did not harm the cell culture system, with the filter paper being cut smaller than the diameter of each well of the culture multiwell dish. As a model, this system can investigate substances released from adhesives with time more conveniently and reliably by using the MTT test and crystal violet staining for testing the viability of cells. The total number of cells remaining corresponds to that following exposure to substances released from polymerized CA that were toxic to the oral fibroblasts. Crystal violet staining is a simple, rapid method used to measure the cytotoxicity of CA adhesives incubated with the media for various periods of time.

The results showed that, even though their cytotoxicity were reduced considerably after incubation with the media for 24 hours, they still released substances that are cytotoxic for at least 2 weeks. This also corresponds with the oral fibroblasts that were subcultured in the dish which had the CA-coated filter paper attached, which developed an inhibitory zone around the CA-coated paper where there no cells appeared to survive, although the distance around the CA-coated filter paper was reduced somewhat with time. There was still reduced zone of inhibition at 2 weeks.

The standard CA that was used to compare cytotoxicity with the commercial CA adhesive is a mixture of ethyl and methyl CA ester, which was the short chain alkyl group of CA. The standard CA's degradation rate is fast and its cytotoxicity is not significantly different from the three commercial CAs, although the standard CA gave a lower percentage of optical density as the control compared to the commercial CAs in the crystal violet staining test.It can be concluded that if this adhesive is used for repair of broken dentures it wil release substances which are toxic to human oral fibroblast cells and these may persist for at least 2 weeks. However this study reflects only in vitro cytotoxicity testing using a cell culture system and suggested only that this commercial adhesive released cytotoxic substances for some time, however the harmful effects of this material on the human system are not conclusive.